Infections of Drosophila melanogaster with pathogens Mycobacterium marinum and Burkholderia thailandensis

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Infections of *Drosophila melanogaster* with pathogens – *Mycobacterium marinum* and *Burkholderia thailandensis*

Thesis submitted for PhD

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ABSTRACT

The first part of this thesis focuses on infection of *Drosophila* with *Mycobacterium marinum*. Tuberculosis remains one of the most widespread infectious diseases in the world affecting approximately one third of the world's population[1]. The bacterium that causes this serious affliction is *Mycobacterium tuberculosis*, a Biosafety Level 3 agent. This bacterium's close relative, *M. marinum*, causes a tuberculosis-like disease in fish and frogs, but does not require special working conditions. In the fruit fly, *Drosophila melanogaster*, *M. marinum* prevents phagosomal acidification, permitting it to survive in fly macrophages. *Drosophila* is thus a genetically tractable model for the study of some stages of tuberculosis. To understand the pathology of *M. marinum* infection in the fly, I have analysed the potential role of several genes mainly by survival assays, quantification of antimicrobial peptide expression, and microscopy. A phenotype emerged in a macrophage-specific knockdown of the *Drosophila* phagocytic receptor Nimrod C3.

The second part of this thesis focuses on infections of *Drosophila* with *Burkholderia thailandensis*. *B. thailandensis* is a Gram-negative bacterium closely related to *Burkholderia pseudomallei*, the causative agent of melioidosis. The study revealed that *B. thailandensis* was pathogenic in the fly; it activated the fly immune system and antimicrobial peptides were expressed. Despite the strong immune response, this infection is lethal and kills *Drosophila* within two days. This result suggests that the bacterium is resistant to antimicrobial peptides; similar findings have been reported in the case of the *B. pseudomallei* resistance to a human antimicrobial peptide *in vitro*.

Overall, this work focuses on host factors involved in immunity to infection and the generation of pathology in intracellular bacterial infections. In each case, we have used pathogens closely related to serious human pathogens, with the aim of identifying conserved mechanisms of pathogenesis and immunity. Along the way, I have generated several experimental tools that will be useful both for the study of these specific infections and for the analysis of infection biology more generally.

[1] *Tuberculosis Fact sheet N°104*. *World Health Organization*. November 2010. Available from: http://www.who.int/mediacentre/factsheets/fs104/en/index.html [accessed 7 Sep 2011].

TABLE OF CONTENTS

ABBREVIATIONS

- ΔT6SS-(1-6) - *B. thailandensis* lacking all five Type VI secretion systems
- ΔT6SS-5 - *B. thailandensis* lacking Type VI secretion system number 5
- AMP - antimicrobial peptide
- AttA - Attacin-A
- *B. pseudomallei* - *Burkholderia pseudomallei*
- *B. thai* - *Burkholderia thailandensis*
- *B. thailandensis* - *Burkholderia thailandensis*
- BF - bright field
- BSL - Biosafety Level
- CD36 - Cluster of Differentiation 36
- CFU - colony-forming unit
- CM - *B. thailandensis*-conditioned medium
- *crq* - *croquemort*
- *D. melanogaster* - *Drosophila melanogaster*
- Def - Defensin
- Dpt - Diptericin
- Dro - Drosocin
- Drs - Drosomycin
- Dscam - Down syndrome cell adhesion molecule
- DsRed - nuclear Discosoma sp. Red fluorescent protein
- *E. coli* - *Escherichia coli*
- eGFP - enhanced green fluorescent protein
- eYFP - enhanced yellow fluorescent protein
- GAL4 - a yeast transcription factor
- h - hour(s)
- *He* - *Hemese*
- *Hml* - *Hemolectin*
- ind. exp. - independent experiments
- k/d - knockdown

- Loctite - cyanoacrylate-based glue
- *M. marinum - Mycobacterium marinum*
- *M. smegmatis - Mycobacterium smegmatis*
- *M. tuberculosis - Mycobacterium tuberculosis*
- min. - minimum
- *Mm - M. marinum*
- mRFP - monomeric red fluorescent protein
- Mtk - Metchnikowin
- NI - untreated
- *nim - nimrod*
- p.i. - post-infection
- PBS - phosphate buffered saline
- *pes - peste*
- pHrodo - pH-sensitive rhodamine-based dye conjugated to dead *E. coli*
- qRT-PCR - quantitative reverse transcription fluorescence PCR
- RpL1 - Ribosomal protein L4
- SD - standard deviation
- *shf - shifted*
- *Snmp - Sensory neuron membrane protein*
- *Sr-C - Scavenger receptor class C*
- UAS - Upstream Activation Sequence; GAL4 binding sequence
- VDRC - Vienna *Drosophila* RNAi Center
- WT - wild-type

Chapter 1. GENERAL INTRODUCTION

1.1. *Drosophila* and immunity

The field of *Drosophila* immunity was preceded by studies on various insects and other species. Elie Metchnikoff (Ilya Ilyich Mechnikov) is considered to be the founder of cellular immunology for his discovery of phagocytic cells (Cooper *et al*., 2002). While he was studying the origin of digestive organs in the starfish bipinnaria larvae, he discovered 'moving cells' that were capable of 'ingesting' foreign objects, such as sharp splinters. Metchnikoff went on to study the progress of infection in the planktonic crustacean *Daphnia pulex* (water flea), and found cells that ingested microbes. The water flea offered an advantage over the starfish; it was possible to study all the stages of development under the microscope because of its transparent body. Metchnikoff named the cells phagocytes, 'devouring cells' in Greek. In 1908, he jointly received the Nobel Prize in Physiology or Medicine, with Paul Ehrlich[2]. His discovery and further work created a basis for the future identification of phagocytes in insects. One example was presented in 1934; phagocytosis of foreign objects, such as ink or carmine granules, was observed in the larvae of the greater wax moth (*Galleria mellonella*) (Cameron, 1934).

Around the time when Metchnikoff received his Nobel Prize, Thomas Hunt Morgan was studying *Drosophila* in relation to Mendelian inheritance. Morgan and his students captured wild-type fruit flies, bred them, and tried various ways to mutagenise them. Eventually, the first mutant fly emerged; it had white eyes instead of red. Further

[2] Mechnikov, I., 1908. *On the Present State of the Question of Immunity in Infectious Diseases – Nobel Lecture*. Available from: http://www.nobelprize.org/nobel_prizes/medicine/laureates/1908/mechnikov-lecture.html [accessed 05 Nov 2011].

crosses to this single white-eyed male resulted in Morgan's first publication on sex-related inheritance in *Drosophila melanogaster* (Morgan, 1910; Sturtevant, 1913). A few years later, Morgan's students Alfred Henry Sturtevant and Calvin Blackman Bridges published their work; Sturtevant constructed the first genetic map of a chromosome and mapped *Drosophila* genes, such as *white* and *vermilion*, that were discovered in Morgan's lab[3] (Sturtevant, 1913); Bridges studied chromosomal non-disjunction and heredity, and contributed to *Drosophila* genetics with the discovery of salivary gland polytene chromosomes. These chromosomes increase in size as a result of DNA replication, but no cell division occurs (Bridges, 1916a, b, 1935). In 1933, Morgan received the Nobel Prize in Physiology or Medicine for his research on fruit flies, and for discovering the role of chromosomes in inheritance (Morgan, 1934)[4]. Although the work of Morgan and his students was not related to immunity, they established *Drosophila melanogaster* as the primary animal model for the study of the nature of genes.

Metchnikoff discovered phagocytosis; Morgan established the fruit fly as a scientific 'tool', and Hans Gustaf Boman brought together the field of immunology and *D. melanogaster*. Boman and colleagues showed that *Drosophila* immune response was induced by bacterial infection. They were interested to know if immunisation would protect *D. melanogaster* from other infections, so they 'vaccinated' adult flies with pathogenic bacteria and then injected the vaccinated flies with a strain of bacteria that was either the same as that used in the 'vaccine' or different. What they found was that only pathogenic bacteria induced protection from a subsequent infection. When testing two related strains of an insect bacterial pathogen, *Pseudomonas aeruginosa* (*P. aeruginosa*), they found that the infected flies were protected from the second strain, yet

[3]Weiner, J., 2000. *Time, Love, Memory*, published by Faber and Faber, 2nd edition.
[4]Morgan, T. H., 1933. *Thomas H. Morgan – Nobel Lecture*. Available from: http://nobelprize.org/nobel_prizes/medicine/laureates/1933/morgan-lecture.html [accessed 29 Jun 2011].

the first 'vaccination' strain continued to grow inside the fly. This bacterial growth was tested in untreated and 'vaccinated' flies, and quantified by plating homogenised samples onto selective agar plates. Although the 'vaccinated' flies were protected from a second infection, the protection was not strain-specific. Additionally, haemolymph obtained from 'vaccinated' flies was more efficient at killing bacteria *in vitro* than that of normal flies. In a later study, Boman and colleagues obtained similar results in larger insects, the silk moth pupae (*Samia cynthia*). These findings proved that infection in the fly and other insects induced the synthesis of a substance that could kill bacteria, the future antimicrobial peptides (Boman *et al*., 1972; Boman *et al*., 1974).

There are many other scientists who have made great contributions to the field of infection and immunity; however, I will mostly refer to the work of the scientists who used *Drosophila* in their research in the context of immunity. In the next few sections, I will attempt to give a concise overview of the *Drosophila* immune system: the recognition of microbes by the fly immune cells, the process of phagocytosis, the immune signalling pathways, and the subsequent expression of antimicrobial peptides.

1.1.1. Plasmatocytes: fly macrophages

Firstly, I would like to briefly compare *Drosophila* plasmatocytes and mammalian macrophages from the point of their function in the respective systems. *Drosophila* haemocytes and mammalian macrophages express phagocytic receptors, and both are professional phagocytes (Abrams *et al*., 1992; Evans *et al*., 2003; Franc *et al*., 1996; Franc *et al*., 1999a; Pearson and Lux, 1995).

Haemocytes were shown to express various scavenger and phagocytic receptors. One such scavenger receptor is Croquemort; it belongs to the family of scavenger receptor type SR-CI, and is homologous to the mammalian CD36 scavenger receptor

(Abrams *et al*., 1992; Franc *et al*., 1996; Franc *et al*., 1999a; Pearson and Lux, 1995; Rämet *et al*., 2002). In the fly, phagocytosis mediated via Croquemort is important in the removal of apoptotic cells and pathogens, such as *Escherichia coli* (*E. coli*) or *Staphylococcus aureus* (*S. aureus*) (Franc *et al*., 1996; Franc *et al*., 1999a; Stuart *et al*., 2005). In mammals the CD36 scavenger receptor is expressed on many cells, but looking just at the macrophage – here it mediates phagocytosis (Silverstein and Febbraio, 2009). In a study using human macrophages, CD36 scavenger receptor had been shown to participate in the phagocytosis of apoptotic neutrophils (Savill *et al*., 1992). In subsequent studies of CD36-mediated phagocytosis, it transpired that this receptor participates in phagocytosing lipids, namely low-density lipoprotein, and this process contributes to the formation of atherosclerotic plaque in mammals (Silverstein and Febbraio, 2009). Although there is no equivalent of blood vessels and atherosclerotic plaques in *Drosophila*, haemocytes are capable of phagocytosing lipids (K. Woodcock, unpublished data), but relevance of these findings must be clarified.

Secondly, fly and mammalian macrophages are to some extent similar from a developmental point of view. Fly plasmatocytes, develop and disperse in two waves, first during the embryonic stages, and second during larval development (Tepass *et al*., 1994). *Drosophila* does not possess an exact counterpart to the vertebrate bone marrow or yolk sac, from which vertebrate macrophages originate (Schulz *et al*., 2012). However, the fly larval lymph gland has been shown to be a haematopoietic organ (Minakhina and Steward, 2010).

In addition, the fly embryo has motile haemocytes; the larva has sets of circulating and sessile; the pupa has sets of circulating cells responsible primarily for remodeling the larva into an adult; and in the adult only sessile cells were observed so far (Elrod-Erickson *et al*., 2000; Holz *et al*., 2003; Lanot *et al*., 2001; Zettervall *et al*., 2004). The

fly macrophages are motile only at some stages of development; this physiological property is somewhat comparable to that of mammalian macrophages, which are differentiated from motile patrolling monocytes, but once differentiated remain within a given tissue (Tacke and Randolph, 2006).

The roles of transcription factors involved in haematopoiesis show genetic similarities in *Drosophila* and vertebrates. One example is Serpent, which is the fly GATA transcription factor required for the cell fate specification of haemocytes. In mice, the Serpent homologues GATA-1, -2, and -3 transcription factors are important during various stages of haematopoiesis, including that of cell specification during early blood cell development (Evans *et al*., 2003).

Realistically, the fly and mammalian macrophages are different cells because they "service" evolutionary distant organisms. However, as discussed above, fly haematopoiesis resembles in many ways embryonic phases of vertebrate haematopoiesis (Evans *et al*., 2003).

1.1.2. Recognition of microbes and phagocytosis

Unlike mammals, *D. melanogaster* does not have any known adaptive immunity, and relies solely on its innate immune system. Before the *Drosophila* immune system can respond to a bacterial, fungal or viral challenge, the pathogens must be recognised by the cells of the immune system. In the fly, these cells are haemocytes and they express specialised recognition molecules that detect the presence of pathogens (Hoffmann and Reichhart, 2002; Kimbrell and Beutler, 2001).

The Gram-negative bacteria-binding protein gene family is an example of such recognition molecules. One member of this family, the Gram-negative bacteria-binding

protein 1 (GNBP-1) is expressed in all developmental stages of the fly, and recognises cell wall fragments from Gram-negative bacteria, lipopolysaccharides (LPS), and fungal β-1,3-glucan (Kim, 2000). Both LPS and β-1,3-glucan are known to trigger an immune response. Overexpression of GNBP-1 results in an increase of the AMP Cecropin A expression after challenge with LPS or β-1,3-glucan (Kim, 2000).

Another example of recognition molecules is the members of the peptidoglycan recognition protein (PGRP) family. One of the *Drosophila* PGRPs, peptidoglycan recognition protein LC (PGRP-LC), has various isoforms that are specific to different bacteria. The PGRP-LCx isoform is important for the detection of Gram-positive bacteria and the presence of a particular peptidoglycan moiety specific for the bacteria, whereas Gram-negative bacteria require the PGRP-LCx and PGRP-LCa isoforms for their detection *in vitro* (Kaneko *et al*., 2004; Leulier *et al*., 2003; Werner *et al*., 2003; Werner *et al*., 2000). The silencing of the *PGRP-LC* gene, using RNAi, leads to severe reduction of AMP expression in response to *E. coli*, a Gram-negative bacterium, and to reduced phagocytosis of *E. coli*, but not *S. aureus*, a Gram-positive bacterium (Rämet *et al*., 2002).

Precise distinction between the host itself and harmful microbes is an indispensable and conserved feature of the immune system across species, not only in *D. melanogaster* (Kang *et al*., 1998). Many receptors for the recognition of pathogens have varied functions to enable the recognition of diverse pathogens, and are expressed on the fruit fly phagocytes, plasmatocytes.

1.1.2.1. Phagocytosis of pathogens and apoptotic cells

Drosophila 'blood' (haemolymph) contains several classes of 'blood cells' (haemocytes). The haemocyte classes are plasmatocytes, lamellocytes and crystal cells.

Lamellocyte differentiation is induced during the larval stages in immune challenge; crystal cells mediate the process of melanisation, usually in response to injury. However, the predominant type of haemocyte is the plasmatocyte, a macrophage-like cell (Franc *et al*., 1999a; Rizki and Rizki, 1980). (In the results chapters of this thesis, the use of plasmatocyte and haemocyte is interchangeable and refers to the adult *Drosophila* plasmatocyte.) Phagocytosis or encapsulation is an essential function to prevent pathogens from spreading, and to ensure healthy development and homeostasis (Hay *et al*., 1994; Lemaitre and Hoffmann, 2007; Rizki and Rizki, 1980).

In the moth species *Galleria mellonella,* haemocytes together with LPS induce the larval fat body to release 'bactericidal' factors *in vitro*. This suggests that haemocytes interact with LPS, and produce a signal that induces the fat body to secrete 'bactericidal' factors (De Verno *et al*., 1984).

Drosophila haemocytes express various phagocytic and scavenger receptors; however, only a few representative receptors will be discussed. The scavenger receptor, **Croquemort** (crq), is an example of a receptor important in the phagocytosis of apoptotic corpses and *S. aureus* (Franc *et al*., 1996; Franc *et al*., 1999a; Stuart *et al*., 2005). It is homologous with the human Cluster of Differentiation 36 (CD36) receptor, which also plays a role in apoptosis (Franc *et al*., 1996; Franc *et al*., 1999a). **Draper** is a scavenger receptor homologous with the nematode *Caenorhabditis elegans* CED-1 receptor. Draper is expressed by glial cells and haemocytes and was shown to be involved in phagocytosis of apoptotic corpses (Freeman *et al*., 2003; Manaka *et al*., 2004). In contrast to Croquemort and Draper, the *Drosophila* scavenger receptor family, class CI (**SR-CI**) possesses similarity with invertebrate and vertebrate proteins that play a role in immunity. This SR-CI class was found to be haemocyte-specific and was

required for the phagocytosis of Gram-positive and Gram-negative bacteria *in vitro* (Pearson and Lux, 1995; Rämet *et al*., 2001).

A mutation in the fly gene *picky eater* (*picky*) affects the function of PGRP-SC1a, a receptor that belongs to the peptidoglycan recognition protein family. This mutation functionally manifests as a defect in phagocytosis of *S. aureus*. However, phagocytosis of other bacteria is not affected, hence the name *picky eater* (Garver *et al*., 2006).

A transmembrane protein **Eater** is important for the phagocytosis of bacteria. A knocked down expression of Eater in *Drosophila* S2 cells leads to a reduction in the phagocytosis of bacteria (Kocks *et al*., 2005). Eater can bind to live or dead Gram-positive bacteria; however, it can bind to Gram-negative bacteria only if the bacterial cell wall has been disrupted. Eater can also bind to *E. coli* that had been 'pre-killed' with the fly antimicrobial peptide Cecropin A (CecA), and to live *E. coli* that had been exposed to CecA. These results led to the proposal that AMPs might work by revealing ligands for the recognition by Eater (Chung and Kocks, 2011). Eater shares sequence similarity with proteins belonging to the Nimrod C family (Kocks *et al*., 2005).

The **Nimrod family** contains several classes of proteins – Nimrod A, Nimrod B1 - B5, Nimrod C1 - C4. Nimrod C1 (NimC1) is a phagocytic receptor expressed on *D. melanogaster* plasmatocytes. Reduced expression of NimC1 in S2 cells results in reduced phagocytosis of bacteria, particularly *S. aureus*. Overexpression of NimC1, on the other hand, leads to increased phagocytosis of *S. aureus* and *E. coli* (Kurucz *et al*., 2007).

The importance of haemocytes was also shown in relation to viral infection. If *D. melanogaster* phagocytosis is blocked by bead injections, thus simulating the depletion of haemocytes, flies infected with Cricket Paralysis virus have significantly reduced survival (Costa *et al*., 2009).

Phagocytosis of pathogens or apoptotic corpses depends on the function of various classes of phagocytic or scavenger receptors that are expressed on the surface of *Drosophila* phagocytic cells – the plasmatocytes.

1.1.3. Signalling immune pathways

Drosophila pathogens induce appropriate responses depending on their characteristics. Gram-negative bacteria, such as *E. coli*, preferentially induce the Imd signalling pathway (De Gregorio *et al*., 2002; Lemaitre *et al*., 1995a; Rämet *et al*., 2002), whereas Gram-positive bacteria, such as *Micrococcus luteus* (*M. luteus*), and fungi, such as *Aspergillus fumigatus*, mostly induce the Toll pathway (Lemaitre *et al*., 1996).

Following the recognition of pathogens and phagocytosis, the next step in *Drosophila* immune response is the induction of immune signalling pathways, Toll, Imd, JNK and/or Jak-STAT. The induction of signalling pathways results in the expression of AMPs or other responses, such as wound healing or apoptosis. The Toll and Imd are pattern-recognition signalling pathways that induce the expression of *D. melanogaster* AMPs in response to infection [Figure 1.1]. AMPs are synthesized and secreted by the fat body (Lemaitre and Hoffmann, 2007).

1.1.3.1. Toll pathway

This immune pathway is named after its main component, the Toll transmembrane receptor, which was discovered in *D. melanogaster* through its role in the formation of dorsal-ventral embryonic polarity (Hashimoto *et al*., 1988). The Toll pathway is activated in response to fungi and Gram-positive bacteria, such as *M. luteus* (Lemaitre *et al*., 1996; Michel *et al*., 2001).

During infection in adult flies, the Toll signalling pathway is activated via the proteolytic cleavage of Spätzle (Spz), a circulating cytokine-like polypeptide, by Spätzle-processing enzyme (SPE). A proteolytically cleaved form of Spz then binds and

activates the Toll receptor (Michel *et al*., 2001). The activated Toll receptor forms a complex with Pelle (kinase), and Tube and MyD88 (adaptor proteins) (Lemaitre and Hoffmann, 2007). In an uninduced state, a NF-κB-related protein, dorsal-related immunity factor (DIF), interacts with its inhibitor protein, Cactus, a homologue of the mammalian IκB (Geisler *et al*., 1992; Nicolas *et al*., 1998). The interaction between DIF and Cactus prevents the over-activation of the Toll target genes; the gain-of-function mutation in *Toll* or the loss-of-function *cactus* mutants formed melanotic tumours (Lemaitre *et al*., 1995b). During infection, Cactus is degraded and DIF translocates into the nucleus, and thus the expression of the antifungal AMP Drosomycin is induced (Ip *et al*., 1993; Lemaitre *et al*., 1995b; Lemaitre *et al*., 1996). The cytoplasmic domain of the *Drosophila* Toll receptor is homologous to that of the human interleukin-1 receptor (Schneider *et al*., 1991). The fly Toll receptor is highly homologous to the human Toll (Medzhitov *et al*., 1997).

1.1.3.2. Imd pathway

The Imd signalling pathway was named after a mutation, the *immune deficiency* (*imd*), which caused an immune phenotype (Lemaitre *et al*., 1995a). Survival of *imd* mutants infected with a mixture of *E. coli* and *M. luteus* was reduced in comparison to wild-type (WT) controls. This study also revealed that the expression of Drosomycin, an antifungal AMP, was not affected in the *imd* mutant, while the antimicrobial peptide such as Diptericin was induced. Based on this result, Lemaitre and colleagues proposed that a different signalling pathway controlled the induction of Drosomycin than that of Drosocin and Diptericin (Lemaitre *et al*., 1995a).

The binding of bacterial peptidoglycan to the transmembrane peptidoglycan recognition protein receptor PGRP-LC or PGRP-LE activates the Imd pathway (Choe *et*

al., 2002; Gottar *et al*., 2002; Kaneko *et al*., 2006; Rämet *et al*., 2002). The PGRP receptors signal downstream via the Imd protein, which shares similarity with receptor interacting protein (RIP), which is associated with tumor necrosis factor signalling and apoptosis (Georgel *et al*., 2001). The activation of the Imd pathway leads to a signalling cascade involving the TAK1 kinase, *Drosophila* inhibitor-of-apoptosis protein 1 (DIAP1), adaptor protein FADD, the Dredd caspase, and a member of the NF-κB family, the transcription factor Relish (Lemaitre and Hoffmann, 2007). The signalling cascade leads to the phosphorylation and cleavage of Relish. The C-terminal fragment of Relish, Rel-49, remains in the cytoplasm, while the N-terminal fragment of Relish, Rel-68, translocates to the nucleus and induces transcription of antimicrobial peptides, such as Drosocin and Cecropins (Hoffmann and Reichhart, 2002; Lemaitre and Hoffmann, 2007; Wiklund *et al*., 2009). The Imd pathway is primarily responsive to Gram-negative bacteria, such as *E. coli*; however it was found to have some effect in the infection by the Cricket Paralysis virus (CrPV), which causes lethal infection in WT *Drosophila*.

The CrPV infection does not trigger AMP expression in flies, but depletes Hml^{+ve} haemocytes (Costa *et al*., 2009). Injecting beads prior to infections with CrPV to block phagocytosis, renders infected flies more susceptible than controls. The authors tested loss-of-function mutants in various components of the Imd pathway, such as *PGRP-LC, Tak1, ird5, key* or *rel*. These mutants displayed not only reduced survival when infected with CrPV, but also increased bacterial load (Costa *et al*., 2009). However, loss-of-function mutants in other components, the *imd* or *Drosophila FADD* gene, did not show the same phenotype; they were not susceptible to the CrPV infection, and their bacterial load was not as significantly higher than that in controls (Costa *et al*., 2009). Altogether this study showcases the complexity of the immune pathway signalling, and the fact that the mechanism of CrPV–Imd pathway interaction is not clear.

1.1.3.3. JAK/STAT and JNK pathways

Apart from the Toll and Imd pathways, the JAK/STAT and JNK signalling pathways are also involved in fly immunity. It has been shown that the JAK/STAT (janus kinase/signal transducers and activators of transcription) pathway is induced in response to tissue damage, septic injury and other stresses by the fly cytokine, Unpaired 3, which is classed as a fly cytokine, and is expressed by haemocytes (Lemaitre and Hoffmann, 2007; Sluss *et al*., 1996). In addition to its role in responses to bacteria, the JAK/STAT pathway offers some anti-viral protection in *D. melanogaster*, specifically in response to *Drosophila* C virus (DCV). The survival of DCV-infected *hopscotch* mutants was reduced in comparison to WT flies; the *hopscotch* gene encodes tyrosine kinase JAK (Dostert *et al*., 2005; Zettervall *et al*., 2004). The role of the JAK/STAT pathway in immunity is not well understood.

As for the JNK (jun-N-terminal kinase) pathway, it is triggered by the activated Imd pathway, and is involved in wound healing and response to injury and other stresses (Boutros *et al*., 2002; Galko and Krasnow, 2004; Rämet *et al*., 2002; Silverman *et al*., 2003; Stronach, 2005). Galko and colleagues investigated the effect of JNK pathway on epidermal healing of the fly larva. They inhibited the JNK pathway by expressing a dominant-negative form of the JNK kinase Basket specifically in the larval epidermis. Following an injury that was either a small puncture wound or large sterile injury to the epidermis, healing in flies with inactivated Basket was disrupted (Galko and Krasnow, 2004).

Additionally, JNK pathway was shown to be involved in AMP induction (Delaney *et al*., 2006; Silverman *et al*., 2003). Specifically, a mutation in the JNK pathway

component TAK1 kinase abolished expression of Relish-dependent AMPs, namely Diptericin, Attacin, Metchnikowin, and Drosomycin (Delaney *et al*., 2006).

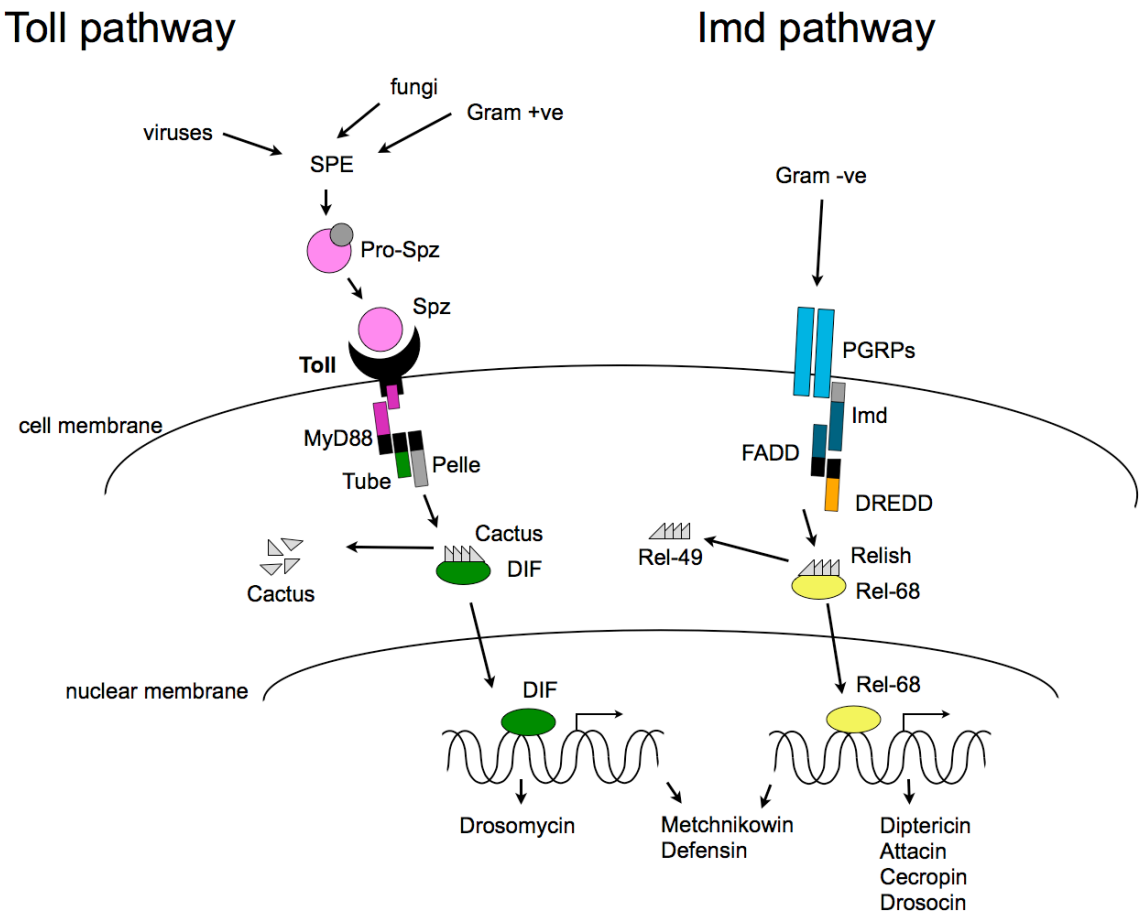

Figure 1.1 **The Toll and Imd signalling pathways.** Infection caused by Gram-positive bacteria, fungi or viruses leads to the proteolytic cleavage of the Toll receptor ligand, Spätzle (Spz) by Spätzle-processing enzyme (SPE). The activated transmembrane Toll interacts with the adaptor protein MyD88, which forms complex with the adaptor protein Tube, and activates the kinase Pelle. As a result Cactus, a DIF repressor and fly homolog of the mammalian IκB, is degraded, and the NF-κB protein DIF, translocates to the nucleus, and induces AMP expression. The Imd pathway is triggered by Gram-negative bacteria; fragments of bacterial peptidoglycans bind to specific PGRP receptors. Activated receptor recruits the Imd protein, which binds to the Fas-associated death domain-containing protein (FADD). FADD recruits the caspase Death related ced-3/Nedd2-like protein (DREDD). This caspase then cleaves Relish, an NF-κB protein, into two fragments; Rel-49, which remains in the cytoplasm, and Rel-68, which translocates into the nucleus, binds DNA and induces AMP transcription.

1.1.4. *D. melanogaster* antimicrobial peptides

In *D. melanogaster*, as in many other insects, antimicrobial peptides (AMPs) are effective against a broad spectrum of bacteria, fungi and viruses. AMPs are produced by the fat body, fatty tissue distributed in the whole body. In the adult *D. melanogaster*, the fat body is found in the head, thorax and abdomen (Dunn *et al*., 1985; Lemaitre and Hoffmann, 2007). The *Drosophila* fat body was described as functionally analogous to the mammalian liver, because the transcriptional activator of the mammalian liver could regulate the Alcohol dehydrogenase (Adh) expression in the fat body of adult fruit flies (Falb and Maniatis, 1992). *Adh* gene is normally expressed by the mammalian liver, and in the adult *D. melanogaster* mostly by the fat body (Sofer, 1987).

The location of the antimicrobial peptide synthesis was studied in the larvae of the tobacco hornworm moth (*Manduca sexta*). The *Manduca sexta* fat body was isolated, cultured *in vitro* and stimulated with peptidoglycan fragments obtained from *M. luteus* cell wall. This experiment verified that insect antimicrobial peptides were secreted by the fat body (Dunn *et al*., 1985). In *Drosophila*, the fat body, and to a lesser extent haemocytes, can secrete the AMPs Cecropins (Samakovlis *et al*., 1990).

AMPs are secreted into the haemolymph, the fly equivalent of blood. Since the circulatory system of *D. melanogaster* is open, the secreted AMPs spread rapidly throughout the *D. melanogaster* body (Lemaitre and Hoffmann, 2007). Some AMPs are expressed constitutively, such as Drosomycin in the salivary glands of the adult *D. melanogaster* (Tzou *et al*., 2000). However, I am going to focus on the expression of AMPs in response to infection only.

In order to be effective against pathogens without damaging the host tissue, AMPs need to be specific for pathogens and foreign bodies. Difference in specificity was

shown using giant silk moth (*Hyalophora cecropia, H. cecropia*) antimicrobial peptide - Cecropin A (CecA) - and bee venom protein - melittin. Both CecA and melittin killed *E. coli*; however, melittin also lysed human-derived Chang liver cells (Steiner *et al*., 1981). AMPs target and kill bacteria and fungi by a variety of mechanisms, for example by forming pores in the bacterial cell membrane. The *Drosophila* AMP Cecropin A (CecA) and bee melittin are examples of peptides forming transmembrane pores in bacterial cell walls. Other AMPs interfere with bacterial protein synthesis, e.g. fruit fly Drosocin (Brogden, 2005).

Cecropins, initially known as the P9 proteins, were the first *Drosophila* AMPs to be sequenced and characterised. They had first been discovered in the pupae of *H. cecropia* (Hultmark *et al*., 1980). *D. melanogaster cecropin* genes *cecA1* and *cecA2* have an identical sequence with the *cecropin* gene sarcotoxin IA of the flesh fly (*Sarcophaga peregrina*), which proves that the insect AMPs are conserved (Kylsten *et al*., 1990; Okada and Natori, 1985). The *Drosophila* AMPs CecA1 and CecA2 are expressed strongly in response to bacterial infection; however, they were also detected in untreated larvae and adults of *D. melanogaster* (Samakovlis *et al*., 1990). On the other hand, the CecB and CecC are expressed in *Drosophila* pupae during metamorphosis, and this suggests that gut bacteria released during metamorphosis triggers AMP expression (Samakovlis *et al*., 1990; Tryselius *et al*., 1992). Cecropins have an antibacterial effect against Gram-negative and Gram-positive microbes and fungi (Lemaitre and Hoffmann, 2007).

Attacins, another class of insect AMPs previously known as immune proteins P5, were also first discovered in the pupae of *H. cecropia* (Pye and Boman, 1977). The *Drosophila Attacin* gene was isolated and characterised using polymerase chain reaction (PCR) as the new method of isolating genes in a whole organism (Åsling *et al*., 1995;

Liang and Pardee, 1992). Attacin is thought to interfere with the synthesis of several bacterial outer membrane proteins and is active against Gram-negative bacteria (Carlsson *et al*., 1991; Lemaitre and Hoffmann, 2007).

Diptericins were first purified from the northern blowfly (*Phormia terraenovae*) (Dimarcq *et al*., 1988). The *Phormia terraenovae* and *D. melanogaster diptericin* genes are very similar (Wicker *et al*., 1990). Following infection with the Gram-negative bacterium *Erwinia carotovora*, Diptericin is expressed in the *Drosophila* respiratory and digestive tract (Tzou *et al*., 2000). The function of the *D. melanogaster* AMPs Diptericins is known to be effective primarily against Gram-negative bacteria, such as *E. coli* (Lemaitre and Hoffmann, 2007).

The *D. melanogaster* AMP **Drosocin** was described and characterised as the first inducible fruit fly antimicrobial peptide, and it is known to be effective against Gram-negative bacteria (Bulet *et al*., 1993; Lemaitre and Hoffmann, 2007).

Defensin was first studied in the cell cultures of *Sarcophaga peregrina* (Matsuyama and Natori, 1988). The *Drosophila* AMP Defensin, similar to Cecropin and Diptericin, was induced without challenge during the pupal stage (Dimarcq *et al*., 1994; Samakovlis *et al*., 1990; Wicker *et al*., 1990). *D. melanogaster* Defensin is known to be active against Gram-positive bacteria; however, it has been shown recently that Defensin was induced in response to Gram-negative bacterium, *Erwinia carotovora*, in the fly genitalia (Dimarcq *et al*., 1994; Gendrin *et al*., 2009).

The **Drosomycin** AMP is effective against various types of fungus. Interestingly, the fly *drosomycin* gene sequence is homologous to that of an antifungal peptide isolated from the seeds of a cabbage family *Brassicaceae* (Fehlbaum *et al*., 1994). The human peptide Drosomycin-like defensin (DLD) is homologous with *D. melanogaster*

Drosomycin and was shown to be active against fungus *Aspergillus fumigatus* (Simon *et al*., 2008).

The *D. melanogaster* **Metchnikowin** is induced in response to fungi and Gram-positive bacteria, such as *M. luteus*. Like the other AMPs, it is expressed primarily in the *D. melanogaster* fat body. The name Metchnikowin was proposed in honour of Elie Metchnikoff (Levashina *et al*., 1995).

Antimicrobial peptides are not limited to insects; the first predicted 'bactericidal' protein PYL[a] was obtained and partially sequenced from the skin of the African clawed frog (*Xenopus lavis*) (Hoffmann and Richter, 1983). The first class of vertebrate AMPs to be discovered was the amphibian magainin family isolated from the *Xenopus lavis* skin (Zasloff, 1987). This added to the excitement and strengthened the hypothesis that the link between the insect humoral response is somewhat conserved in other animal classes, such as vertebrates. The research into antimicrobial peptides continued and a human AMP was discovered in the respiratory system, tracheal antimicrobial peptide (TAP). Later, human cells, keratinocytes and enterocytes, were shown to express AMPs (Diamond *et al*., 1991; Zasloff, 2002).

In recent years, expression of *D. melanogaster* AMPs has been mostly used as a tool – a read-out of immunity-related activity. For instance, the *Drosophila* fusion proteins Drosocin-GFP and Defensin-GFP were used to study tissue-specific expression in larvae and adult flies in response to bacteria (Tzou *et al*., 2000).

1.2. *Drosophila* tools

Drosophila-derived cell lines

Although cell lines of *D. melanogaster* and other Dipteran species had previously been obtained and studied, one of the crucial advances was made in 1972 by Imogene Schneider; she developed an improved procedure of obtaining and successfully keeping cultured cells derived from embryonic stages of *D. melanogaster*. In her study, Schneider described three lines of spontaneously immortalised cells: 1. having diverse morphology, such as spindle-shaped or 'macrophage-like'; 2. having morphology like that of epithelial cells and growing in a monolayer; and 3. aggregating and not growing in a monolayer (Schneider, 1972).

The *Drosophila* embryo-derived cell lines have been popular for *in vitro* experiments as they offer many advantages over other cell lines, (Boutros *et al*., 2004; Clemens *et al*., 2000; Kleino *et al*., 2005; Rämet *et al*., 2001). One of the main advantages is the ease of loss-of-function experiments using RNA-induced gene silencing (RNAi) (Rogers and Rogers, 2008). The main types of *Drosophila*-derived cell lines are the S2 cells (Schneider's line 2), and its derivatives, such as the S2R+ cells; the S2 cell line has phagocytic properties and is thought to be derived from *D. melanogaster* haemocytes (Rämet *et al*., 2002).

Genetics

D. melanogaster has four pairs of chromosomes: one pair of sex chromosomes and three pairs of autosomes. The third pair of autosomes is very small, encoding approximately 1% of the genome, and is usually ignored. The remaining three pairs of

chromosomes have been widely studied. Balancer chromosomes are one of the invaluable research tools of *Drosophila* genetics. Balancers are chromosomes with many inversions, which prevent homologous recombination, and thus allow the maintenance of specific mutations on that particular chromosome. In addition, balancers contain recessive lethal and dominant visible markers that give rise to specific phenotypes and allow the identification of the required mutant flies. The presence of at least one recessive lethal permits the maintenance of the desired genotype[5].

Another tool, the GAL4-UAS system, allows for directed transgene expression in *D. melanogaster*. GAL4 is a transcription factor that comes from yeast, and so is exogenous to *D. melanogaster*. The second half of this system is a GAL4 binding sequence - Upstream Activation Sequence (UAS). To make this system useful and specific, UAS is positioned before the gene of interest (Brand and Perrimon, 1993; Duffy, 2002). GAL4, under the control of a defined, tissue-specific or ubiquitous driver, binds the UAS and as such drives the expression of the gene of interest that is under the control of the UAS. For example, to examine the expression of the fly gene *Hemolectin* (*Hml*), GAL4 would be under the control of the *Hml* promoter, and the UAS would be controlling the expression of a fluorescent protein, such as GFP. If both elements, GAL4 and UAS, are expressed in the same fly, the system is active and genes can be silenced (by driving dsRNA or dominant-negatives) or overexpressed, and specific populations of cells can be killed or made to express a fluorescent protein. However, different transgenic fly stocks can carry each element of this system, and can be crossed when the GAL4-UAS system needs to be implemented. The GAL4-UAS system thus allows for controlled expression of target genes (Brand and Perrimon, 1993; Duffy, 2002).

[5] Greenspan, R. 2004. *Fly Pushing: The Theory and Practice of Drosophila Genetics*, published by Cold Spring Harbor Laboratory Press, 2nd edition.

1.3. Mycobacteria

Mycobacterium tuberculosis (*M. tuberculosis*) is a human pathogenic bacterium that causes tuberculosis. Robert Koch discovered and described *M. tuberculosis*. In 1882, he presented his data to the Physiological Society of Berlin, and later, received the Nobel Prize in Physiology or Medicine[6] (Schultz, 2011).

Tuberculosis is among the most important infectious diseases in the world. The World Health Organization reports that approximately 1.7 million people died of the disease in 2009 alone[7]. Despite its importance, we understand comparatively little about the pathogenic mechanisms of this infection. In part, this is because the causative agent of this infection, *M. tuberculosis,* is difficult to study: it is dangerous, requiring that all work be done at Biosafety Level 3, and it grows very slowly, so that the time from infection to death can take several months in mice (Radaeva *et al*., 2005). However, *M. tuberculosis* is closely related to *Mycobacterium marinum* (*M. marinum*) (Tønjum *et al*., 1998), which grows faster in experimental models (Clark and Shepard, 1963; Radaeva *et al*., 2005), and does not cause systemic disease in people, and thus does not need to be handled in a special environment. *M. marinum* is a natural pathogen of fish and frogs and causes tuberculosis-like disease in these animals (Ramakrishnan *et al*., 1997); in the fruit fly *Drosophila melanogaster, M. marinum* causes an infection which is similar in many ways to *M. tuberculosis* infection in humans (Dionne *et al*., 2003). The genetic tractability of *Drosophila*, combined with the ease of working with *M. marinum* compared to *M. tuberculosis*, makes this model an interesting one for examining the interactions between pathogenic mycobacteria and their hosts.

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[6] Koch, H. H. R., 1905. *Robert Koch - Nobel Lecture* [online]. Available from: http://nobelprize.org/nobel_prizes/medicine/laureates/1905/ [accessed 2 Jul 2011].
[7] *Tuberculosis Fact sheet N°104*. World Health Organization. November 2010. Available from: http://www.who.int/mediacentre/factsheets/fs104/en/index.html [accessed 7 Sep 2011].

Various types of pathogenic mycobacteria cause mycobacterial infections in humans and other animals: the most common of these are tuberculosis (caused by *M. tuberculosis*) and leprosy (caused by *Mycobacterium leprae*). Mycobacteria belong to a diverse bacterial family of the phylum Actinobacteria (Ventura *et al*., 2007). Actinobacteria are classed as Gram-positive, but mycobacteria, together with Corynebacteria and Nocardia, also belong to a sub-group of alcohol and acid-fast bacteria. The cell wall of alcohol and acid-fast bacteria contains mycolic acid, a lipid substance that gives the bacterial cell wall a 'waxy' characteristic. This particular type of cell wall makes the bacteria hardy, and resistant to antibiotics and diagnostic stains. As a result, though they are phylogenetically Gram-positive, they do not stain with Gram stain, and special histological staining methods are necessary to identify this type of bacteria (Ellis and Zabrowarny, 1993).

Phagocytes play a vital role during mycobacterial infection. Normally, macrophages ingest pathogens and destroy them by acidifying the internal environment of the phagocytic vesicle. However, many kinds of pathogens, including pathogenic mycobacteria, have evolved the ability to modify the phagocytes to their own advantage so that they can reproduce inside them without destruction (Barker *et al*., 1997; Hagedorn and Soldati, 2007). Live and dead *M. marinum* were processed differently by mouse macrophage cell line (RAW 264.7); dead *M. marinum* co-localised with the vacuolar proton ATPase, while live bacteria did not (Barker *et al*., 1997). Hagedorn and colleagues observed similar phenomenon using the professional phagocyte *Dictyostelium discoideum* as a model. Their data shows that dead or dying *M. marinum* co-localised with phagolysosomal markers, Lysobisphosphatidic acid, Lysosome-associated membrane protein 1, and flotillin-1, whereas endosomal compartments containing live and proliferating bacteria contained only flotillin-1 and not the other examined components (Hagedorn and Soldati, 2007).

Thus, the pathogenic mycobacterium shelters from the effects of the humoral response and evades being killed by the mature phagolysosome.

M. marinum is related to the human pathogen *M. tuberculosis*

Drosophila melanogaster was established as a tractable model host for *M. marinum* infection: not only is *M. marinum* lethal to *Drosophila*, but some stages of this infection in the fly also resemble those of *M. tuberculosis* infection in people (Dionne *et al.*, 2003). Mycobacteria are slow growing bacteria consisting of many strains that cause similar diseases in diverse hosts, such as fish, cattle, and humans; *M. tuberculosis* is a serious pathogen in humans. Mycobacteria are special due to their cell surface, which has a thick mycolate-rich coat that serves as a protective barrier, and is difficult to penetrate. This mycolate-rich coat also contains many compounds involved in virulence.

M. marinum is closely related to *M. tuberculosis* phylogenetically; Tønjum and colleagues studied the relationship of different mycobacteria, including *M. marinum* and *M. tuberculosis*. Using DNA-DNA hybridization and 16S rRNA analysis they showed that *M. marinum* is closely related to *M. tuberculosis* (Tønjum *et al.*, 1998).

From the point of view of infection, *M. marinum* and *M. tuberculosis* are also closely related. Phagocytic cells process the bacteria in the same manner, and the course of *M. marinum* in its natural hosts, such as fish and frogs, resemble *M. tuberculosis* infection in humans (Ramakrishnan *et al.*, 1997). Pathogenic mycobacteria proliferate in the host's macrophages, the very cells responsible for the killing and degradation of bacteria (Dionne *et al.*, 2003). Live *M. marinum* bacteria are processed by mouse macrophages in a similar way as *M. tuberculosis* (Barker *et al.*, 1997; Clemens and Horwitz, 1995). Mycobacteria can live in a diverse range of professional phagocytes, such as those of the mouse or zebrafish, and in amoeba (*Dictyostelium discoideum*) (Clay *et al.*, 2007; Hagedorn and Soldati, 2007; Pozos *et al.*, 2004; Swaim *et al.*, 2006).

Infection studies proved that *M. marinum* injected into the fruit fly causes infection similar to the course of infection caused by *M. tuberculosis* in humans (Dionne *et al*., 2003).

The main signalling pathway through which the *Drosophila* immune system responds to mycobacteria is unknown. Human Toll-like receptors (TLRs) respond to mycobacteria and elicit an immune response (Brightbill *et al*., 1999); however, evidence suggests that the Toll and Imd mediated pathways are not involved in responding to mycobacterial infection in *Drosophila (Dionne et al., 2003)*.

1.4. *Burkholderiaceae*

The *Burkholderia* bacteria consist of a large group of human, animal and plant pathogenic and non-pathogenic species (Coenye and Vandamme, 2003). Species such as *Burkholderia cepacia*, *Burkholderia thailandensis, Burkholderia mallei,* or *Burkholderia pseudomallei* are extensively studied because of the danger that they pose to healthy and especially immunocompromised people (Currie *et al*., 2000a; Suputtamongkol *et al*., 1999; White, 2003). Immunocompromised individuals, who suffer from chronic diseases such as cystic fibrosis or diabetes, are mainly in danger of an infection by *Burkholderia cepacia* (*B. cepacia*) or *Burkholderia pseudomallei (B. pseudomallei*) (Coenye *et al*., 2001; McClean and Callaghan, 2009; Suputtamongkol *et al*., 1999; White, 2003). In cystic fibrosis patients, respiratory infection caused by *Burkholderia cepacia* causes complications and increases the risk of death; diabetes sufferers have much more increased rate of death if infected by *B. pseudomallei*. Unlike *B. cepacia* or *B. thailandensis*, *Burkholderia mallei* (*B. mallei*) and *B. pseudomallei* are also capable of causing disease in healthy individuals (White, 2003; Whitlock *et al*., 2007). *B. pseudomallei* infection - melioidosis - has wide ranging symptoms and as many as 40% of patients who receive full antibiotic treatment die of melioidosis (White, 2003). *B. mallei* causes glanders in horses and can cause serious infection in people, but this infection does not occur often (Whitlock *et al*., 2007). This is perhaps due to the fact that *B. mallei* is an obligatory zoonotic pathogen and does not survive outside its host (Nierman *et al*., 2004). However, *B. mallei* together with *B. pseudomallei* are listed as Category B critical biological agents by the US Centers for Disease Control and Prevention due to their potential of spreading rapidly and causing high mortality (Rotz *et al*., 2002).

In this thesis, I am concerned only with two of the *Burkholderia* species: *B. pseudomallei* and *B. thailandensis*. *B. pseudomallei* is an intracellular, motile, Gram-negative bacterium that has been documented to be capable of "invading" mammalian cells (macrophages) *in vitro* (Stevens *et al*., 2003). *B. pseudomallei* can infect via broken skin or the respiratory system (White, 2003). Melioidosis is a dangerous malady that is highly endemic in areas of Southeastern Asia and Northern Australia (Chaowagul *et al*., 1989; Cheng and Currie, 2005; Smith *et al*., 1995), and endemic in other regions such as India and southern China (Currie *et al*., 2008). Melioidosis can manifest as acute septicaemia, but also as a chronic infection (Chaowagul *et al*., 1993; White, 2003).

The effect and progress of *B. pseudomallei* infection was studied in Syrian golden hamsters and mice (Brett *et al*., 1997; Stevens *et al*., 2004). Other studies focused on the life cycle of *B. pseudomallei* and its resistance to antimicrobial peptides *in vitro*, or to antibiotic treatment of patients (Brett *et al*., 1997; Dance *et al*., 1989; Jenney *et al*., 2001; Pilatz *et al*., 2006; Vorachit *et al*., 1993). However, work with *B. pseudomallei* requires BSL-3 containment (Rotz *et al*., 2002). The other avenue is to use *B. thailandensis*, which closely related to *B. pseudomallei*, as a model organism (Brett *et al*., 1998; Kim *et al*., 2005; Yu *et al*., 2006). Although *B. thailandensis* is considered non-pathogenic in mammals, high doses of *B. thailandensis E264* kill mice (Haraga *et al*., 2008; Wiersinga *et al*., 2008), and *B. thailandensis* infections in people have been documented to cause melioidosis-like symptoms, but this is very rare (Glass *et al*., 2006; Lertpatanasuwan *et al*., 1999).

The fly has been used extensively as a model for the study of viral and bacterial infections such as the *Drosophila* C virus, *Mycobacterium marinum* or *Staphylococcus aureus* (Dionne *et al*., 2003; Dostert *et al*., 2005; Needham *et al*., 2004). In comparison

with microbes such as *Listeria monocytogenes*, *Salmonella typhimurium* or *M. tuberculosis*, many aspects of *B. pseudomallei* pathology are not yet understood.

Techniques that can be utilised in studying infection in the fly are survival assay, analysis of AMP expression of WT flies or immunocompromised mutants survival (Dionne *et al*., 2003; Hedengren *et al*., 1999; Rämet *et al*., 2002; Rutschmann *et al*., 2000). Although the fly has no known adaptive immunity, it is an attractive potential model host to examine the role of innate immunity in melioidosis.

The goal of this study was to establish whether the innate immunity of *D. melanogaster* could be a suitable model for the study of *Burkholderia*-related pathogenicity.

1.5. Modelling pathogenic infections in *D. melanogaster*

D. melanogaster is a tractable model for the study of host-pathogen interactions using various bacteria, such as *P. aeruginosa*, *M. marinum* or *Salmonella typhimurium* (*S. typhimurium*) (Brandt *et al*., 2004; D'Argenio *et al*., 2001; Dionne *et al*., 2003).

D. melanogaster was exploited as a model to screen mutants of the bacterium *P. aeruginosa*, which is pathogenic not only in the fly and other insects, but also in plants and humans. The use of *D. melanogaster* as a model permitted the discovery of a mutant strain of *P. aeruginosa* that killed the fly slower in comparison to the control bacterial strain. Thus, the fly as a simple model was useful in examining virulence factors of a versatile pathogen, *P. aeruginosa* (D'Argenio *et al*., 2001). *D. melanogaster* offers the possibility of screening large samples of to test mutant bacteria in an *in vivo* system.

M. marinum infection is lethal in *D. melanogaster*, and the early stages of pathogenesis arising from this infection in the fly resemble the known early stages of human tuberculosis. The phagocytosed *M. marinum* prevents the normal course of phagosome maturation; it prevents vacuolar acidification of *Drosophila* plasmatocytes in a similar fashion as it does in mammalian macrophages (Barker *et al*., 1997; Dionne *et al*., 2003).

The innate and adaptive branches of the mammalian immune system work together and this is very complex, so dissecting the importance of individual molecules is difficult. The fruit fly lacks adaptive immunity, which is a limitation from the point of view that results cannot be directly translated into higher organisms such as mice or humans. However, from a different perspective, the fly innate immune system offers advantages, such as simplicity, some gene conservation, advanced genetic tools, short

time of propagation, cheap infection assays, and fast response of the organism to infection. Notwithstanding, the fly is an ethically comfortable animal model. *Drosophila melanogaster* as a model will not help answer all the questions, but is sophisticated enough to help scientists to draw nearer to the answers.

1.6. Thesis outline

The main focus of my work has been on the interactions of pathogenic bacteria with the host. The model organism, the host in this case, was *Drosophila melanogaster*. I have used adult male *Drosophila* for *in vivo* assays and *Drosophila* embryo-derived cell lines for *in vitro* experiments. The next section, chapter 2, provides an account of materials and methods that I used in my work.

My PhD project changed several times, which is the reason why the results chapters are written as individual units. In chapter 3, I discuss the analysis of the gene *shifted*, which seemed to be important in *M. marinum* infection of the fly. This project was based on preliminary results - fruit flies with a mutation in the *shifted* gene were long-lived after an infection with *M. marinum*.

In chapter 4, I describe an imaging method of infection of live adult *D. melanogaster*. I developed a technique that allowed the imaging of immobilised, not anaesthetised, live flies. The aim of this project was to test this technique to image the progress of *M. marinum* infection.

In chapter 5, I describe results obtained from a screen to reveal defects or advantages gained by *D. melanogaster* with knockdown expression in the genes of interest in relation to *M. marinum* infection. The knockdown of these genes was always haemocyte-specific. The *nimrod C3* (*CG16880*) gene, in particular, had a phenotype in connection with *M. marinum* infection. Haemocyte-specific knockdown of *nimrod C3* resulted in a lower bacterial load and lower expression of the antimicrobial peptide Metchnikowin.

The results described in chapter 6 were obtained using a novel *D. melanogaster* pathogen – *Burkholderia thailandensis*. The purpose of this project was to explore the possibility of using the fruit fly as a model host for this infection, and try to advance our understanding of the involvement of the innate immune system in *Burkholderia thailandensis* pathogenicity of *D. melanogaster*. This bacterium is a close relative to a human pathogen and causative agent of melioidosis, *Burkholderia pseudomallei*.

Chapter 2. MATERIALS AND METHODS

2.1. Fly stocks

- *c564-GAL4*, fat body-specific driver (Hrdlicka *et al*., 2002), from the Bloomington *Drosophila* Stock Center; Stock no: 6982; on chromosome 2
- Cluster of Differentiation 36 (CD36) family
- *UAS-CG10345.IR*, ***CG10345***, from VDRC: Transformant ID 100252; Library KK; on chromosome 2
- *UAS-CG1887.IR*, ***CG1887***, from VDRC: Transformant ID 100219; Library KK; on chromosome 2
- *UAS-CG2736.IR*, ***CG2736***, from VDRC: Transformant ID 102672; Library KK; on chromosome 2
- *UAS-CG3829.IR*, ***CG3829***, from VDRC: Transformant ID 103492; Library KK; on chromosome 2
- *UAS-CG7227.IR*, ***CG7227***, from VDRC: Transformant ID 108059; Library KK; on chromosome 2
- *UAS-pes.IR*, ***peste*****,** CG7228, from VDRC: Transformant ID 100391; Library KK; on chromosome 2
- *UAS-Snmp1.IR*, ***Sensory neuron membrane protein 1*****,** CG7000, from VDRC: Transformant ID 104210; Library KK; on chromosome 2
- *UAS-Snmp2.IR*, ***Sensory neuron membrane protein 2*****,** CG7422**,** from VDRC: Transformant ID 101136; Library KK; on chromosome 2
- *crq-GAL4*, ***croquemort*** (crq), CG4280, haemocyte-specific driver (Franc *et al*., 1996), from the Dionne lab stocks, on chromosome 3

- *crq-GAL4*, on chromosome 2
- *crq-GAL4*, *UAS-CD8-cherry*, on chromosome 3
- *crq-GAL4*, *UAS-myr-mRFP*, on chromosome 3
- crq RNAi line (*UAS-crq.IR*), from VDRC: Transformant ID 45884; Library GD; on chromosome 3
- *da-GAL4*, ***daughterless*** (*da*), ubiquitous driver (Gaumer *et al*., 2000), from the Bloomington *Drosophila* Stock Center; Stock no: 8641; on chromosome 3
- DrosDel isogenic background w^{1118}
- *He-GAL4*, ***Hemese*** (*He*), CG31770, from VDRC, on chromosome 3
- *HmlΔGAL4*, ***Hemolectin*** (*Hml*), CG7002, on chromosome 2
- *HmlΔGAL4, UAS-2xeGFP*, on chromosome 2, kind gift from Sergey Sinenko[8]
- Scavenger receptor (Sr) family class C
    - *UAS-Sr-CII.IR*, ***Scavenger receptor class C, type II***, CG8856, from VDRC: Transformant ID 100928; Library KK; on chromosome 2
    - *UAS-Sr-CIII.IR*, ***Scavenger receptor class C, type III***, CG31962, from VDRC: Transformant ID 102716; Library KK; on chromosome 2
    - *UAS-Sr-CIV.IR*, ***Scavenger receptor class C, type IV***, CG3212, from VDRC: Transformant ID 100487; Library KK; on chromosome 2
- *Tub-GAL4*, ***α-Tubulin*** (Tub), CG1913, is a ubiquitous driver (Matthews *et al*., 1989; O'Donnell *et al*., 1994), on chromosome 3
- *Tub-GAL80^{TS}*, temperature-sensitive GAL4 repressor (Lee and Luo, 1999), from the Bloomington *Drosophila* Stock Center; Stock no: 7017; on chromosome 3
- *UAS-2xeGFP*, on chromosome 2
- *UAS-2xeYFP*, on chromosome 2, or 3

[8] Sinenko, S.A., Mathey-Prevot, B., 2004. Increased expression of Drosophila tetraspanin, Tsp68C, suppresses the abnormal proliferation of ytr-deficient and Ras/Raf-activated hemocytes. Oncogene 23, 9120-9128.

- *UAS-Apoliner* (a kind gift of the Jean-Paul Vincent lab), on chromosome 1, 2, or 3.
- *UAS-Dscam-like.IR*, ***Down syndrome cell adhesion molecule-like***, *CG32387*, from VDRC: Transformant ID 36287; Library GD; on chromosome 2
- *UAS-Dscam1.IR*, ***Down syndrome cell adhesion molecule 1***, *CG17800*, from VDRC: Transformant ID 108835; Library KK; on chromosome 2
- *UAS-Dscam3.IR*, ***Down syndrome cell adhesion molecule 3***, *CG31190*, from VDRC: Transformant ID 6685; Library GD; on chromosome 2
- *UAS-DsRed2.Nuc21*, on chromosome 2
- *UAS-DsRed2.Nuc22*, on chromosome 3
- *UAS-eater.IR*, ***eater***, *CG6124*, from Bloomington *Drosophila* Stock Center: Stock no: 25863; on chromosome 3
- *UAS-eCFP-bAct*, on chromosome 2
- *UAS-myr-mRFP*, on chromosome 2, or 3
- *UAS-nimA(1).IR*, ***nimrod A***, *CG42282*, from Vienna *Drosophila* RNAi Center (VDRC): Transformant ID 105009; Library KK; on chromosome 2
- *UAS-nimA(2).IR*, ***nimrod A***, *CG42282*, from VDRC: Transformant ID 104204; Library KK; on chromosome 2
- *UAS-nimC1.IR*, ***nimrod C1***, *CG8942*, from Bloomington *Drosophila* Stock Center: Stock no: 25787; on chromosome 3
- *UAS-nimC2.IR*, ***nimrod C2***, *CG18146*, from Bloomington *Drosophila* Stock Center: Stock no: 25960; on chromosome 3
- *UAS-nimC3.IR*, ***nimrod C3*** (in text *nimC3-17054*), *CG16880*, from VDRC: Transformant ID 103668; Library KK; on chromosome 2
- *UAS-nimC3.IR*, ***nimrod C3*** (in text *nimC3*), *CG16880*, from VDRC: Transformant ID 103668; Library KK; on chromosome 2

- *UAS-nimC4.IR*, ***nimrod C4***, *CG16876*, from VDRC: Transformant ID 101915; Library KK; on chromosome 2
- *UAS-shf.IR*, ***shifted*** (*shf*), *CG3135*, from VDRC: Transformant ID 101915; Library KK; on chromosome 2
- wild-type *Oregon R*

Fly crosses - *UAS-p35* and *da-GAL4* or *Tub-GAL4*

UAS-p35 line was crossed to flies carrying one of two ubiquitous drivers, *Tubulin* (*Tub*) or *daughterless* (*da*). The aim was to overexpress p35 ubiquitously to test if this line works well [Figure 4.5]. Appropriate controls were also included.

Cross A: virgins carrying *da-GAL4* driver were crossed to males carrying the transgene *UAS-p35*

Cross B: WT virgins were crossed to *UAS-p35* males (control)

Cross C: *da-GAL4* virgins were crossed to WT males (control)

Cross D: *Tub-GAL4* virgins were crossed to *UAS-p35* males

Cross E: *Tub-GAL4* virgins were crossed to WT males (control)

Eclosed progeny of these crosses was collected and counted at least once per day for the duration of one week.

2.2. *Drosophila* cell cultures

Three different *Drosophila melanogaster* cell lines were used for *in vitro* infections:

1. S2 (S2-DRSC) cell line was derived from late *Drosophila* embryos of wild-type (*Oregon R*) flies, and is a phagocytic, macrophage-like cell line (Foley and O'Farrell, 2004; Rizki and Rizki, 1980; Shields and Sang, 1970).

2. S2-R+ cells, in comparison to S2 cells, express a receptor and are classified as adherent (Yanagawa and Lee, 1998). This cell line was also derived from late *Drosophila* embryos of wild-type (*Oregon R*) flies.

3. $S2^{star}$ cell line originated from S2 cells.

All cell lines were obtained from the *Drosophila* Genomics Resource Centre and cultured in Schneider's Insect Medium (Sigma) supplemented with 10% heat-inactivated FBS (Sigma), GlutaMax (Invitrogen) and penicillin/streptomycin (Invitrogen).

2.3. Bacterial cultures

Cultures of wild-type *M. marinum*, *M. marinum* expressing *DsRed* under the constitutive promoter *msp12* (*msp12::DsRed*) (Clay *et al*., 2007), and *Mycobacterium smegmatis* (*M. smegmatis*), all from the Dionne lab stocks, were cultured from frozen stocks in Middlebrook 7H9 medium (Difco) containing Middlebrook (O)ADC enrichment solution (BD), 0.2% Tween-80 (Sigma), ampicillin (100 μg/ml; Sigma), and nalidixic acid (30 μg/ml; Sigma). In the case of DsRed *M. marinum* kanamycin (30 μg/ml; Sigma) was also added. The cultures were grown at 25 °C for approximately one week. *E. coli* (*DH5α)* and *M. luteus* were cultured from frozen stocks in a standard

sterile LB at 37 °C overnight. *M. marinum* culture was processed into single cell suspension.

Single cell suspension

Single *M. marinum* were separated from clumps of bacteria as follows. A turbid bacterial culture was spun at 3000 x g for 5 minutes at room temperature. The resulting pellet was resuspended in PBS with added 0.2% Tween-80. Next, the cells were spun at 200 x g for 5 minutes at room temperature. This pelleted clumps of bacteria while leaving single bacteria in suspension. The concentration of bacteria was measured using spectrophotometer set to 600 nm for OD determination. To test fly survival, the suspension was subsequently diluted to a desired ratio of 500 CFU/injection ($1x10^7$ CFU/ml). This protocol was adapted from (Gao *et al*., 2003).

Cultures of WT *B. thailandensis E264* (kind gift of Madeleine Moule and Brendan Wren), WT GFP-labelled and T6SS mutant *B. thailandensis* (kind gift from the Mougous lab) (Schwarz *et al*., 2010), T3SS mutant *B. thailandensis* (kind gift from the Miller lab) (Haraga *et al*., 2008) and *Escherichia coli* (*E. coli*) *DH5α* were set up from frozen stocks and cultured in standard lysogeny broth (LB) at 37 °C overnight with agitation. For infection assays with phosphate buffered saline (PBS) as a control, bacterial cultures were harvested by centrifugation at 2400 x g for 4 minutes at room temperature, re-suspended in PBS and calibrated using a spectrophotometer (Eppendorf); for infections with LB as a control, cultures were kept in the original growth medium and calibrated with LB to the desired density. *B. thailandensis* was calibrated to OD_{600} of 0.01, which represents approximately 250 CFU per fly when injected. *E. coli* was calibrated to OD_{600} of 1. To ensure that the LB broth was not

contaminated, separate bacteria-free LB was prepared and treated in exactly the same way as LB containing bacteria.

Heat-inactivated *B. thailandensis* stock was prepared as per Sarkar-Tyson *et al.* (Sarkar-Tyson *et al.*, 2009). The protocol was slightly modified; inactivated cultures were kept as frozen stocks at -80 °C. Heat-killed *B. thailandensis* was tested for viability by incubating in liquid LB at 37 °C for 48 h with shaking.

For experiments with *B. thailandensis*-conditioned medium (CM), overnight cultures were harvested by centrifugation, but in this case the supernatant was removed into a new tube and sterile-filtered using a 0.2 μm filter (Sartorius). To ensure that the CM contained no live bacteria, a portion of the same CM that had been injected into flies was plated on standard LB agar plate and kept at 37 °C for 48 hours. As a control, 5 ml of LB was treated and processed in precisely the same way as the CM, and used for mock-infections as well as for plating.

2.4. Imaging and image processing

For imaging experiments, adult *Drosophila* males the required genotypes were treated in the same way as for survival assays, but were injected with DsRed *M. marinum* or dead *E. coli*-conjugated pHrodo, a rhodamine sensor of pH (pHrodo *E. coli* BioParticles®, Invitrogen). Controls were untreated. Injected and control flies were immobilised with the help of cyanoacrylate-based glue (Loctite), and imaged using a fluorescent (Leica) or confocal microscope (Leica TCS SP5), and capturing software (Leica Application Suite Advanced Fluorescence software). Imaris image processing and analysis software (Bitplane) was used for automated counting of haemocytes in untreated flies.

2.5. Survival and *in vivo* infection assays

Male *D. melanogaster* used for survival assays were kept in 30 ml tubes with roughly 8 ml *Drosophila* medium (recipe as per the Dionne Lab protocols). Eclosed males of the required genotypes were collected from these tubes under light CO_2 anaesthesia once a day, and transferred into tubes containing fresh food. They were allowed to mature for 5-10 days prior to injecting with the required bacteria (see list below). In the case of *M. marinum* infections, mature male flies were injected with a single cell suspension containing 500 or 5000 CFU/ml of bacteria diluted in PBS with 0.2% Tween-80. In the case of *B. thailandensis* infections, calibrated bacterial suspension or sterile filtered *B. thailandensis*-CM was used. Mock-infection control flies were injected with a solution of PBS and 0.2% Tween-80, PBS or LB, and all injections were done using a Picospritzer® III microinjector (Intracel). In most experiments, a third set of untreated males was kept also as a control. Depending on the experiment, the infected and control flies were kept at 18 or 25 °C; dead flies were counted at least once a day.

Bacteria:

- WT *M. marinum*
- DsRed-expressing *M. marinum* (in chapter 4 - abbreviated to *M. marinum*)
- WT M. smegmatis
- WT *B. thailandensis E264* (kind gift of Madeleine Moule and Brendan Wren)
- GFP-labelled *B. thailandensis E264* (kind gift from the Mougous lab)(Schwarz *et al.*, 2010)
- T6SS mutants *B. thailandensis* – number 1-6, and 5 (kind gift from the Mougous lab)(Schwarz *et al.*, 2010)

- T3SS mutants *B. thailandensis* - Bsa mutant AH174, and complemented mutant AH181 kind gift from the Miller lab) (Haraga *et al*., 2008)
- *E. coli* DH5α
- *M. luteus* (kind gift of the Wade lab)

Unless stated otherwise, bacteria were in Dionne lab stocks.

2.6. *M. marinum* bacterial burden in infected *D. melanogaster*

M. marinum-infected *D. melanogaster* (3 males per sample) and controls were collected and homogenised in a solution of chloroform (75 μl or 3 parts, Sigma) and MeOH (25 μl or 1 part, Sigma), and TRIzol reagent (375 μl, Invitrogen) at required time points. mRNA was extracted and cDNA synthesized using the First Strand cDNA Synthesis Kit (Fermentas). The kit was used according to manufacturer's instructions. Random Hexamers were contained in the First Strand cDNA Synthesis Kit, and used for random priming during cDNA synthesis. To quantify *M. marinum* mRNA levels, obtained cDNA was analysed by quantitative RT-PCR using *M. marinum*-specific primers *R8-9* (Dionne *et al*., 2006). Obtained results were analysed using Prism (GraphPad Software).

2.7. *B. thailandensis* load in infected *D. melanogaster*

Infected *D. melanogaster* (1 male per sample) and controls were collected and homogenised in 100 μl of PBS at required time points. One tenth of each sample was processed into a series of dilutions of 1 in 10 in PBS; 4 dilutions were made in total. 10 μl of each incremental dilution was plated on a standard LB agar plate and kept at 37 °C for 24 hours. Bacterial colonies were counted on a light microscope (Nikon). Finally, to obtain the approximate numbers of viable bacteria (CFU) per fly at a given time point of

infection, individual bacterial counts were multiplied appropriately, e.g. the number of colonies obtained from the first dilution (1 in 10) was multiplied by 100. Obtained results were analysed using Prism (GraphPad Software).

2.8. Feeding assays for survival and dissections

Flies were maintained and selected as per survival assays. Overnight culture of *B. thailandensis* was spun at 4 °C at maximum speed for 5 minutes to obtain bacterial pellet. The spent media was removed and the bacteria were resuspended in 1/50x PBS supplemented with 1mM each $CaCl_2$ and $MgCl_2$. Fly food was prepared using dry mix containing 8.5 g fructose (Fruisana), 6.1 g dry milk powder (Marvel), 18 g Smash brand dehydrated mashed potatoes. 1 g of this dry mix was placed into each fly vial and 2 ml of bacterial suspension was added. Control food was prepared using the dry mix and PBS. The fly food was ready to use in less than 30 minutes. Experimental and control flies were put on the appropriate food and counted daily. When flies started to die, at 44 hours of being placed on the infected food, experimental and control flies were taken; their guts were dissected and imaged using fluorescent (Leica) microscope, and capturing software (Leica Application Suite Advanced Fluorescence software).

2.9. mRNA extraction and cDNA synthesis

Total mRNA was extracted from infected and control flies using 100 μl of TRIzol reagent (Invitrogen) as per the manufacturer's protocol. Complementary DNA (cDNA) was synthesized using the First Strand cDNA Synthesis Kit (Fermentas). The kit was used according to the manufacturer's instructions. Random Hexamers were contained in the First Strand cDNA Synthesis Kit, and used for random priming during cDNA synthesis. Obtained cDNA was analysed by quantitative RT-PCR.

2.10. *In vitro* infections and mRNA extractions

Drosophila cell culture medium was replaced with antibiotic-free medium 24 hours prior to infections. Poly-L-lysine (Sigma) was applied to 24 well plates prior to seeding to prevent the loss of S2 cells during exchange of medium by aspiration. At an optimal growth stage of cell culture (5 x 10^6 cells/ml), the bacterial cultures of *E. coli*, *M. luteus*, and *M. smegmatis* were added. The correct density of bacteria was determined using a spectrophotometer (Eppendorf), so that the final ratio was 10 CFU for every S2 cell (10:1). In parallel to infections, a volume of bacteria-free, antibiotic-free cell medium was added to control uninfected cells (mock infections). S2 cells were homogenised in TRIzol reagent (Invitrogen) at required time points. In a few instances, some time points were omitted if amounts of cells were insufficient to carry out an experiment in at least triplicates. mRNA was extracted and cDNA synthesized as above. To quantify *M. marinum* mRNA levels, obtained cDNA was analysed by quantitative RT-PCR using *M. marinum*-specific primers *R8-9* (Dionne *et al*., 2006). Obtained results were analysed using Prism (GraphPad Software).

2.11. Quantitative Reverse Transcription PCR (qRT-PCR)

For quantitative analysis of gene expression, quantitative reverse transcription fluorescence PCR (qRT-PCR) was done using the double-stranded DNA dye SYBR Green (SensiMix, Quantace) in accordance with manufacturer's instructions. The following primer pairs were used:

attacin (AttA, CG10146) sense, 5′-CACAATGTGGTGGGTCAGG-3′, antisense, 5′-GGCACCATGACCAGCATT-3′

croquemort (crq, CG4280) sense, 5′-GCAGATAACCTTGTAGAGGATGG-3′, antisense, 5′-GCAGATAACCTTGTAGAGGATGG-3′

defensin (Def, CG1385) sense, 5′- TTCTCGTGGCTATCGCTTTT-3′, antisense, 5′-GGAGAGTAGGTCGCATGTGG-3′

diptericin (*Dpt*, *CG12763*) sense, 5′-ACCGCAGTACCCACTCAATC-3′, antisense, 5′-CCCAAGTGCTGTCCATATCC-3′

drosocin (Dro, CG10816) sense, 5′-CCATCGAGGATCACCTGACT-3′; antisense, 5′-CTTTAGGCGGGCAGAATG-3′

drosomycin (Drs, CG10810) sense, 5′-GTACTTGTTCGCCCTCTTCG-3′; antisense, 5′-CTTGCACACACGACGACAG-3′

eiger (*egr*, *CG12919*), sense, 5′-GATGGTCTGGATTCCATTGC-3′, antisense, 5′-TAGTCTGCGCCAACATCATC-3′

hemolectin (Hml, CG7002) sense, 5′-CGATGATGACGACGAGGATA-3′, antisense, 5′-GGCTTTGAGGATGTTGAAGC-3′

M. marinum-specific **primers** ***R8-9*** sense, 5′-ACCGCTACGAGGTCAACAAT-3′, antisense, 5′-ATTCGACGAACTCCACCAAG-3′ (Dionne *et al*., 2006)

metchnikowin (Mtk, CG8175) sense, 5′-TCTTGGAGCGATTTTTCTGG-3′; antisense, 5′-TCTGCCAGCACTGATGTAGC-3′

ribosomal protein L4 (RpL1, CG5502) sense, 5′-TCCACCTTGAAGAAGGGCTA-3′; antisense 5′-TTGCGGATCTCCTCAGACTT-3′ (Cho *et al*., 2005; Ranz *et al*., 2001)

shifted (*shf*, CG3135), sense, 5′-AACCCTACTTCACTAACAACTTTGC-3′, antisense, 5′-TGTGGTGCTCGTTGCAGTA-3′

shf-B, sense, 5′-ACGCTGTCCATTCGCAAG-3′, antisense, 5′-CAGGGCAGGAATATGCTAAAA-3′

shf-INT-1A, sense, 5′-CAACGAAACGACTGTTTGTTTTT-3′, antisense, 5′-GCACCAGATATAACCACTTGACC-3′

shifted – new primer set number 6 (*shf*6) - sense, 5′-GGCATCTCGTTGTGGATCA -3′, antisense, 5′-GCCCTGTGGGAAGTAGAGC-3′

unpaired 3 (*upd3*, *CG33542*), sense, 5′-GCAAGAAACGCCAAAGGA-3′; antisense, 5′-CTTGTCCGCATTGGTGGT-3′

The primer pairs were designed using Universal ProbeLibrary (Roche, https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp) to detect the desired gene transcripts, and supplied by Sigma. RpL1 was used as a normalising gene (Cho *et al*., 2005; Ranz *et al*., 2001). qRT-PCR analysis was done using the Rotor-Gene 6000 (Corbett Life Science) and Rotor-Gene 6000 Series Software (Corbett Life Science).

2.12. Statistical tests

Comparison between survival curves was performed using the Log-rank analysis (Mantel-Cox) and the difference was accepted as significant only if the p-value was less than 0.0001. The statistical significance of bacterial growth between time points, and the levels of antimicrobial peptide (AMP) expression were analysed using Mann-Whitney test. All statistical analyses were performed using Prism (GraphPad Software).

Chapter 3. ROLE OF THE *SHIFTED* GENE IN MYCOBACTERIAL INFECTION

Abstract

Mycobacterium marinum is a bacterium related to *Mycobacterium tuberculosis*, a serious human pathogen; therefore, we study *M. marinum* infection in *Drosophila melanogaster* as a model for human tuberculosis.

Based on preliminary data, the fly gene *shifted* (*shf*) was induced in *M. marinum*-infected wild-type (WT) flies, and *shf* mutants lived longer than infected *D. melanogaster*. It is not known how the gene is regulated. Using RNAi the function of transcription factors that were identified via bioinformatics approaches as potential *shf* regulators was knocked down in a tissue-specific pattern. Initially, the expression of *shf* in response to infection was analysed *in vitro* and later studied in whole animals. In parallel, flies carrying a *shf*-IR insertion were crossed to those carrying a tissue-specific driver to knockdown *shf* function in the whole fly, the fat body or haemocytes. The progeny of this cross was infected with *M. marinum* or a non-pathogenic control, *M. smegmatis*. The aim of this study was to discover the regulatory molecular signals of *shifted* induction in *M. marinum*-infected flies, and to study the consequences of *shf* expression in infection. Despite using several tissue-specific drivers and repeating experiments several times, the preliminary data were not confirmed.

3.1. Introduction

The *Drosophila shifted* (*shf*) gene encodes a secreted protein orthologous to the vertebrate Wnt inhibitory factor-1 (WIF-1). WIF-1 is a protein secreted by the Wingless pathway in vertebrates; it is an inhibitor and thus prevents Wnts from signalling. Wnts are signalling molecules important in development in flies and vertebrates, such as the axis formation in Xenopus (McMahon and Moon, 1989; Swarup and Verheyen, 2012). However, the fly WIF-1 ortholog, Shf, cooperates with *Drosophila* Hedgehog protein (Hh), which is an important signalling molecule in development, and enables its stabilisation and diffusion in the developing fly wing (Glass *et al*., 2006; Glise *et al*., 2005; Gorfinkiel *et al*., 2005). The vertebrate WIF-1 binds to *Drosophila* Wingless (Wg), a fruit fly Wnt ligand that is important in the development of segment polarity (Bejsovec and Arias, 1991; Hsieh *et al*., 1999). Although Shf does not seem to be involved in the Wg pathway in the fly development (Gorfinkiel *et al*., 2005), it is unknown if Shf interacts with the *Drosophila* Wg pathway in infection.

shf mutants have developmental defects, one of which gives this mutant its name – *shifted* (Gorfinkiel *et al*., 2005; Morgan and Bridges, 1916). In *shf* mutants, the position of the longitudinal wing vein number 3 (L3) is shifted closer to the longitudinal wing vein number 4 (L4) in comparison to the wild-type [Figure 3.1].

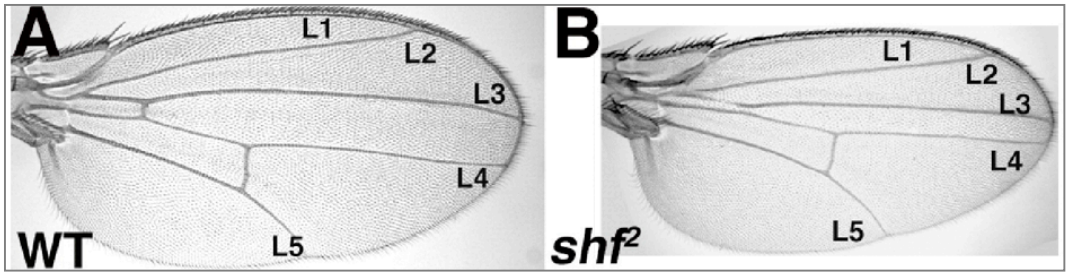


Figure 3.1 **Wing phenotype of *shf* mutants.**
A.) Wild-type wing showing the position of the *Drosophila* longitudinal wing veins (L1-L5).
B.) The position of the longitudinal wing vein L3 in closer to L4 in *shf* mutant. Figure from Gorfinkiel et al.(Gorfinkiel et al., 2005).

The *Drosophila shifted* (*shf*) was included in a screen testing the immune response of various mutants to *M. marinum* infection (Dionne, unpublished data). *shf* mutants had a long-lived phenotype in *Drosophila* infected with *M. marinum*. The goal of this study was to attempt to answer the following questions:

1. How is *shf* regulated?
2. Does Shf interact with Wnts and Hh, or both, during *M. marinum* infection?
3. What role does *shf* play in *M. marinum* infection?

Unfortunately, my data did not support the preliminary results. *M. marinum*-infected tissue-specific *shf* knockdown flies were not significantly longer-lived (nor shorter-lived) in comparison to controls. I was unable to obtain data about *shf* mRNA levels from *in vitro* and *in vivo* infections using qRT-PCR as *shf* primers did not work well.

3.2. Results

3.2.1. Survival assays of *D. melanogaster* with tissue-specific knockdown of *shf*

Preliminary survival data of *M. marinum*-infected *shf* mutants (Dionne, unpublished observations) suggested the involvement of the *shf* gene in *M. marinum* pathology in the fly [Figure 3.2].

D. melanogaster males with tissue-specific or ubiquitous knockdown of *shf* were infected with *M. marinum*, and their survival was compared to WT controls. Three different drivers – *Tubulin* (ubiquitous), *c564* (fat body-specific driver) and *croquemort* (haemocyte-specific) – were used to drive the *shf* RNAi [Figure 3.3]. The survival of the *shf* knockdowns was neither significantly higher nor lower than that of WT controls. Despite the fact that the survival assays were repeated several times (at least three times), the new results did not support the preliminary data [Figure 3.2].

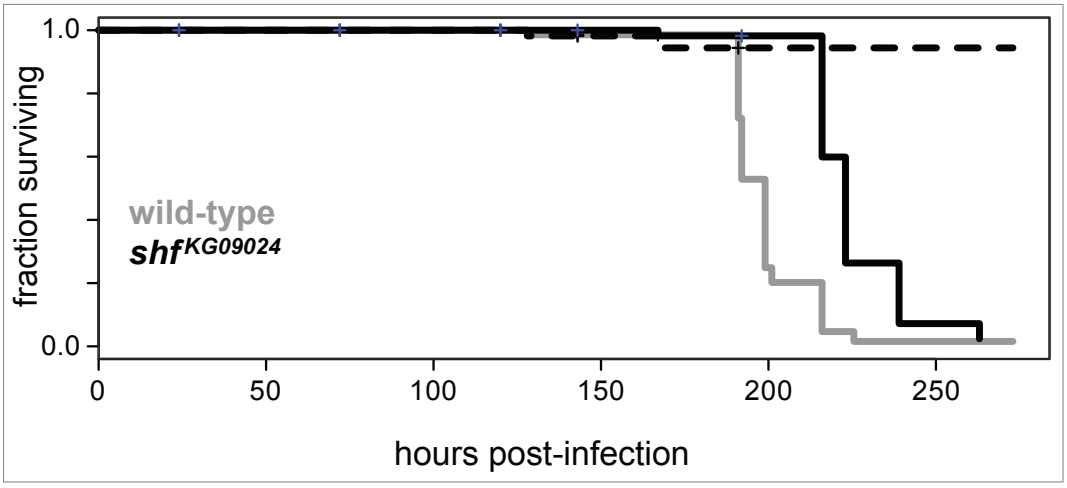


Figure 3.2 **Preliminary data:** *shf* mutants infected with *M. marinum* were longer-lived than WT controls. This survival was repeated at least five times; n = minimum (min.) 100 flies per genotype, per condition.

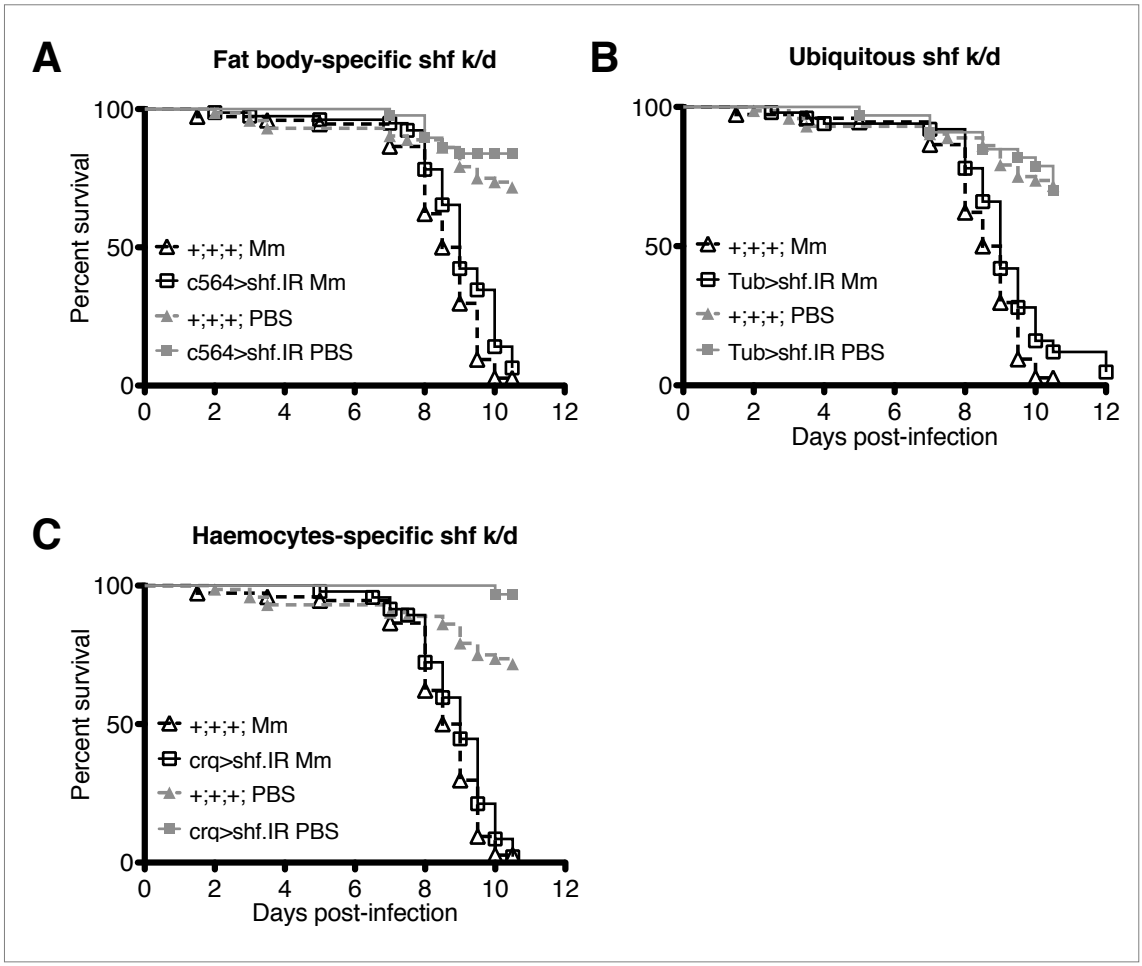


Figure 3.3 **Survival of ubiquitous and tissue-specific knockdown of *shf* does not affect survival in *M. marinum* infection.**
A.) Fat body-specific knockdown using *c564–GAL4*; 3 independent experiments (n = min. 72 flies).
B.) Ubiquitous knockdown using *Tubulin-GAL4*; the survival data is based on at least 2 independent experiments (n = min. 33 flies).
C.) Haemocyte-specific knockdown of *shf* using *crq-GAL4*; data is based on at least 2 independent experiments (n = min. 32 flies).
Difference between survival curves was assessed using Log-rank (Mantel-Cox) test, but no difference was statistically significant.

The fly line carrying the *UAS-shf.IR* transgene was tested using two ubiquitous drivers, *daughterless* (*da*) and *Tubulin* (*Tub*) (Gaumer *et al*., 2000; Matthews *et al*., 1989; O'Donnell *et al*., 1994). Levels of *shf* mRNA were measured using qRT-PCR. In both cases, the *shf* gene was expressed significantly less in comparison to controls, the respondent gene or driver-only controls [Figure 3.4].

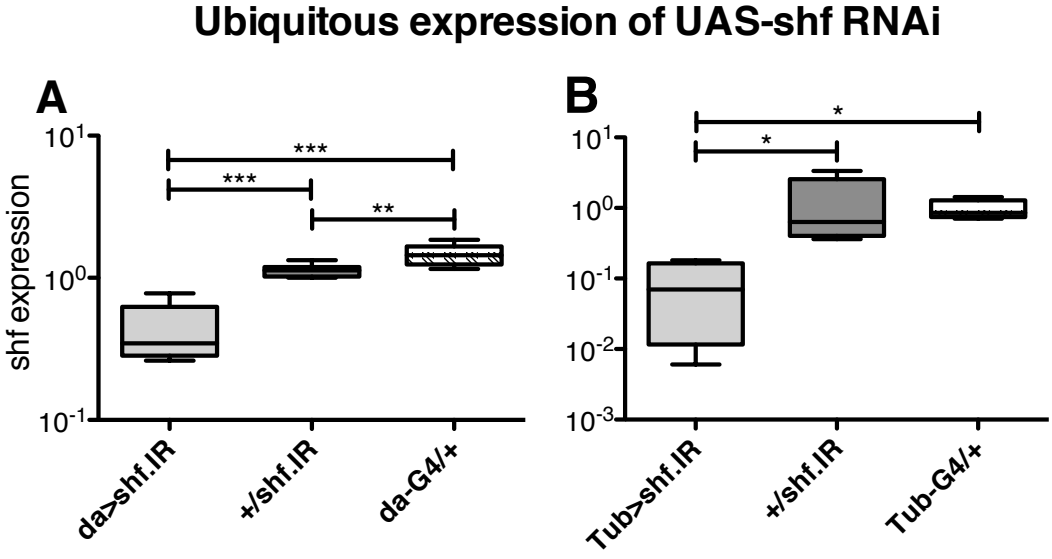


Figure 3.4 **The potential of the *shf.IR* line was tested using ubiquitous drivers, *daughterless* (*da*) and *Tubulin* (*Tub*).** The shf gene expression was significantly lower when RNAi against shf was driven ubiquitously.
A.) *daughterless*-driven knockdown of *shf* (*da>shf.IR*), respondent gene (+/*shf.IR*), and driver-only (*da-G4/+)* controls. Data is based on 1 experiment, n = 8 (24 flies per genotype).
B.) *Tubulin*-driven knockdown of *shf* (*Tub>shf.IR*), respondent gene (+/*shf.IR*), and driver-only (*Tub-G4/+)* controls. Data is based on 1 experiment, n = 4 (12 flies per genotype) in the case of *Tub>shf.IR*; controls n = 5 (15 flies).
Levels of *shf* mRNA were determined by RT-qPCR, and statistical significance was determined using Mann-Whitney test (GraphPad Prism); * $p < 0.02$, ** $p < 0.002$, and *** $p = 0.0002$. Line in boxes represent the median; whiskers 5 – 95 percentile.

3.2.2. *In vitro* infection assay

The aim of this project was to study the induction and regulation of *shf* during mycobacterial infection. *M. marinum* infection was not done *in vitro* because preliminary data (Dionne, unpublished observations) showed that *shf* was induced by *M. marinum* infection in the fly [Figure 3.5].

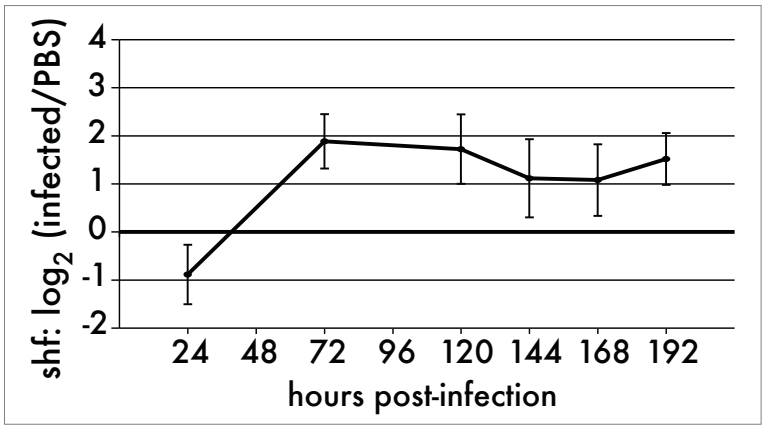


Figure 3.5 **Preliminary data:** *shifted* expression was induced by *M. marinum* infection in WT flies. mRNA levels were measured by qRT-PCR in infected and control males; n = 3 samples (= 9 flies). The data was normalised to PBS-injected controls. Error bars represent SD.

To investigate if *M. marinum* was the only pathogen inducing *shf* expression, *Drosophila* cell cultures (S2, S2*, S2R+) were used for two reasons: 1. it is a simpler model (how useful is that?); 2. these experiments were done at the beginning of my PhD when the fly room was not ready for use, and I could not do experiments with whole animals. The fly cell cultures were infected with several types of bacteria: *M. smegmatis* (non-pathogenic mycobacterium), *M. luteus* (Gram-positive bacterium), and *E. coli* (Gram-negative bacterium). *M. smegmatis* is not normally pathogenic to *Drosophila*, but it was necessary to establish whether *shf* was induced in response to this non-pathogenic mycobacterium. *E. coli* and *M. luteus* were used as representative bacteria of

different classes – Gram negative and Gram positive. Despite the fact that *E. coli* and *M. luteus* do not kill *Drosophila*, immune induction can be observed *in vivo* and *vitro* (Choe *et al*., 2002; Elrod-Erickson *et al*., 2000; Nehme *et al*., 2011).

Following infections, mRNA was extracted from infected and control cells at required time points - 1, 3 or 4, 6 or 6.5, 14 or 18, and 24 hours post-infection (p.i.). cDNA was synthesized, and analysed using qRT-PCR. However, quantification of *shf* mRNA levels proved difficult because *shf* primers did not work reliably (data not shown). In order to confirm that the infection assay worked, I analysed mRNA levels of Metchnikowin, a *Drosophila* AMP. *Metchnikowin* induction was observed in cell culture (mbn-2) 4 hours after LPS was added to the culture; septic injury induced expression of *Metchnikowin* in larvae, pupae and adult flies, in which the expression was strong at 6 hours post-injury (Levashina *et al*., 1995; Levashina *et al*., 1998). These kinetics are in keeping with the published observations of others (De Gregorio *et al*., 2002; Levashina *et al*., 1998). *shf* mRNA was successfully tested by qPCR only twice, using *shf-B* and *shf-int-1A* primers, and only in the *Drosophila* S2* cell line [Figure 3.6 A and C]. Although *shf* does not appear to be induced in response to *E. coli*, the sample size was small to draw definite conclusions. Metchnikowin, used as a positive control, was induced [Figure 3.6B].

cDNA synthesised from cell cultures (S2, S2R+, S2*) infected with *E. coli*, *M. smegmatis*, or *M. luteus*, was further tested for induction of the fly cytokines Eiger and Unpaired 3. Eiger is homologous to the mammalian TNF, and like TNF in mammals, Eiger induces apoptosis in the fly (Igaki *et al*., 2002; Moreno *et al*., 2002). Unpaired 3 is a ligand of the JAK/STAT pathway, and is expressed in haemocytes in response to septic injury (Agaisse *et al*., 2003). Induction of the *unpaired 3* and *eiger* genes was

tested in response to *E. coli*, *M. smegmatis* or *M. luteus* in S2 cells. None of the tested bacteria induced the cytokines (data not shown).

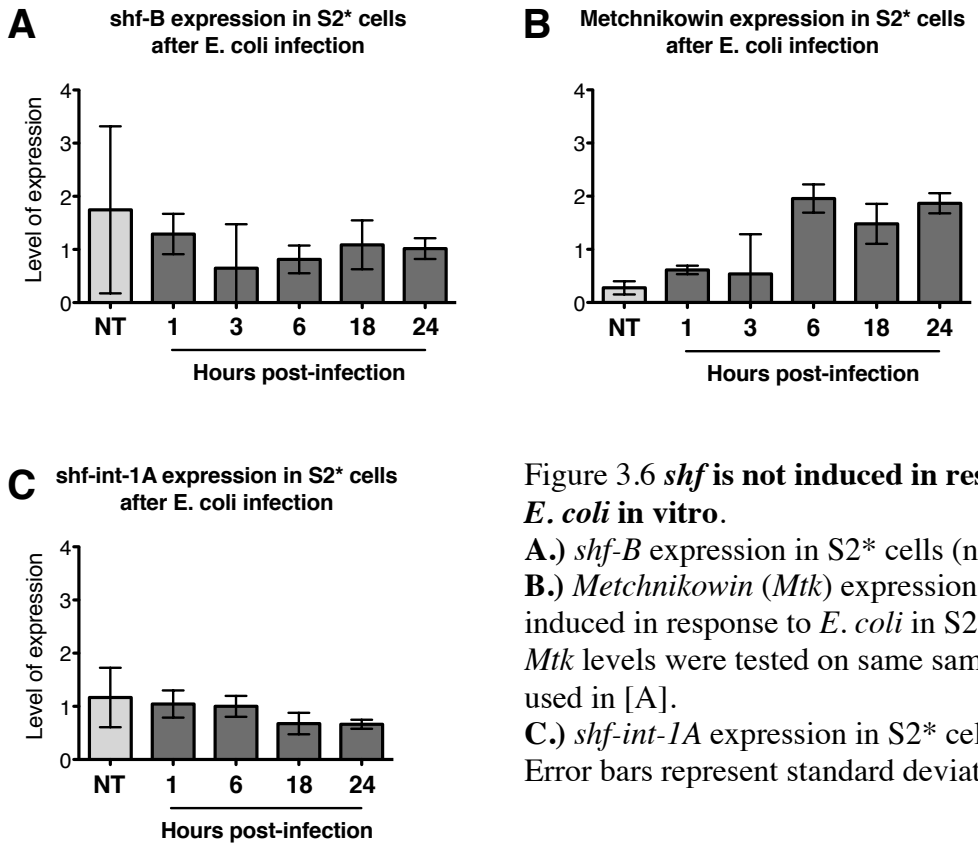


Figure 3.6 ***shf* is not induced in response to *E. coli* in vitro**.
A.) *shf-B* expression in S2* cells (n = 3).
B.) *Metchnikowin* (*Mtk*) expression was induced in response to *E. coli* in S2* cells. *Mtk* levels were tested on same samples as used in [A].
C.) *shf-int-1A* expression in S2* cells (n = 3).
Error bars represent standard deviation (SD).

3.3. Conclusion

Based on preliminary findings (Dionne, unpublished observations), *shf* gene seemed to play a role in *M. marinum* infection. When the gene was mutated or knocked down, *D. melanogaster* lived longer following *M. marinum* infection. Unfortunately, I did not obtain results that would support the preliminary data. Tissue-specific knockdown of *shf* did not increase survival of *M. marinum*-infected flies, even though the knockdown construct was capable of reducing *shf* expression.

After many repeated experiments, and a great deal of consultation, the discrepancy between the preliminary data and my data was most likely due to the difference in fly food. The preliminary data was acquired in a different lab. I spent over a year testing and trying to replicate the preliminary findings. Using mutants and knockdowns, ubiquitous and tissue-specific, of *shf* resulted in a conclusion that the phenotype in *M. marinum*-infected flies was lost. When it became obvious that I could not replicate the preliminary data, I did not complete some experiments related to this topic.

Chapter 4. *IN VIVO* IMAGING OF *M. MARINUM* INFECTION PROGRESS IN ADULT *D. MELANOGASTER*

Abstract

Drosophila melanogaster (*D. melanogaster*) is an accepted model for the study of various infections. The response of the fly immune system can be investigated on several levels – survival, bacterial load, bacterial clearance or proliferation, and expression of *Drosophila* antimicrobial peptides (AMPs) or the lack of it. *In vivo* microscopy can reveal aspects of infection, such as phagocytosis of pathogens by immune cells or the behaviour of a given pathogen in the host, that might not be obvious or easy to assess using other methods. I set out to improve our ability to image the *in vivo* events of mycobacterial infection

I was particularly interested to image the process of phagocytosis and the progress of infection in live adult *D. melanogaster*. The progress of infection was not completed – I managed to image phagocytosed bacteria, but never the actual ingesting of bacteria by the haemocyte. However, I succeeded in imaging the progress of *Mycobacterium marinum* infection. In order to use microscopy on live infected flies, I used an immobilising method that allowed the imaging of the same area of infected flies on at least two occasions 24 hours (h) apart. The goal of this part of the project was to develop techniques to permit sophisticated, high-resolution microscopic analysis of the process of infection.

4.1. Introduction

Imaging of adult *D. melanogaster* macrophages during *M. marinum* infection

In vivo imaging of *Drosophila* macrophages (haemocytes)[9] is possible in embryos; at this stage haemocytes are migratory (Stramer *et al*., 2005; Wood and Jacinto, 2007). In larval stages, haemocytes can be imaged *in vivo* (Márkus *et al*., 2009), but it is more difficult to do so because the larvae move despite being lightly anaesthetised with CO_2 (personal observation, data not shown). In this work, only adult fruit flies were used.

In order to analyse phagocytosis and progress of *M. marinum* infection by microscopy, adult haemocytes were labelled with fluorescent proteins (Franc *et al*., 1999a; Rizki and Rizki, 1980). Haemocyte-specific expression of fluorophores was achieved using the promoter regions of the *Hemolectin*, *croquemort*, *peroxidasin*, or *Hemese* genes as a part of the *UAS-GAL4* system (Brand and Perrimon, 1993; Franc *et al*., 1996; Goto *et al*., 2003; Goto *et al*., 2001; Kurucz *et al*., 2003; Márkus *et al*., 2009; Nelson *et al*., 1994; Stofanko *et al*., 2008; Stramer *et al*., 2005). The expression of some haemocyte-specific genes is limited to certain developmental stages. For example, the *peroxidasin* gene promoter was not used in this study as a driver, because the gene is expressed in embryonic and larval stages, but in adults the expression is detectable only in females (Nelson *et al*., 1994; Stofanko *et al*., 2008; Stramer *et al*., 2005); in male adult flies the expression decreases within three days post-eclosion (K. Woodcock, personal communication). On the other hand, *Hemolectin* and *croquemort* are expressed not only in embryonic and larval stages, but also in adults (Franc *et al*., 1996; Goto *et al*., 2003; Goto *et al*., 2001; Stramer *et al*., 2005).

[9] The terms haemocytes and plasmatocytes are used interchangeably here.

Croquemort belongs to the *Drosophila* CD36 family of scavenger receptors and is expressed in haemocytes (Franc *et al*., 1996; Franc *et al*., 1999a). *Hemolectin* is similar to the mammalian von Willebrand Factor, which is important in maintaining blood homeostasis (Goto *et al*., 2001). *Drosophila* lacks the mammalian components of the blood coagulation cascade, such as platelets, and thus it is likely that the fly haemolymph homeostasis is maintained differently to that of mammals (Goto *et al*., 2001). Nevertheless, the fly body is capable of closing and healing wounds (Galko and Krasnow, 2004; Stramer *et al*., 2005). Larvae with the *Hml* gene silenced, using RNAi in haemocytes or ubiquitously, do not have any developmental defects, but if they sustain injury, such as a stab wound with a fine tungsten needle, the knockdown larvae bleed much more than control flies (Goto *et al*., 2003). In this study, a truncated promoter of the *Hemolectin* gene (*HmlΔ*) was used only as a driver. Nevertheless, survival of *M. marinum*-infected flies expressing eGFP under the control of *HmlΔ* was tested and was not significantly different from that of wild-type (WT) flies [Figure 5.7 A].

Microscopy is in many cases not sufficient as a sole tool for the study of different aspects of infection, but can offer insight into many cellular processes, such as how the process of phagocytosis is affected by infection and how the infection spreads. Active infection due to proliferating bacteria expressing GFP was shown in the fly using *P. aeruginosa*, *M. marinum*, and *S. aureus* previously (Dionne *et al*., 2003; Fauvarque *et al*., 2002; Needham *et al*., 2004).

In this study the progress of *M. marinum* infection in the fly was imaged with microscopy, to determine the method's suitability as a tool for the visual study of infection *in vivo*.

4.2. Results

4.2.1. *In vivo* imaging of adult *Drosophila* haemocytes

After my attempt at understanding the role of the *shifted* gene in *M. marinum* infection, imaging infection and phagocytosis was an appealing direction to me. Initially, I learnt the optical projection tomography (OPT) technique in an attempt to image whole flies (McGurk *et al*., 2007). The OPT technique generated 3D images of adult flies (males and females) expressing exogenous ß-Galactosidase (ß-Gal). As expected, the level of X-gal staining of control (non-ß-Gal expressing) flies was fainter than that of ß-Gal-expressing flies, but the difference was not clear-cut and staining of adult flies was not reliable, partly because of poor diffusion of reagents through the adult cuticle and partly because of the heavy pigmentation of the adult fly.

Next, I used fluorescent and confocal microscopy to image fluorescent haemocytes of untreated WT males; however, even anaesthetised flies moved slightly, making it difficult to perform time-lapse imaging or even to image the same location in different individuals. To solve this problem, I tested several techniques to immobilise flies without killing them. First, I tested agarose gel 'semi-embedding', which allowed the immobilised fly to breathe and also prevented it from dehydrating. Before using this method on infected flies, I tested the 'semi-embedding' on uninfected WT flies. My goal was to image the exact location of infected flies at several time points to show the potential bacterial proliferation. I attempted to keep the flies alive in the agarose for 24 hours, keeping the immobilised flies in a makeshift humid 'chamber' to prevent the agarose from drying and shrinking. However, the humid 'chamber' did not prevent the agarose from shrinking, so the immobilised flies escaped when kept overnight. This was unlikely to be a safe method for immobilising infected *D. melanogaster*.

After the agarose 'semi-embedding' method failed, I tested other, more vigorous adhesives, such as heptane glue, which is used in securing live *Drosophila* embryos for imaging[10]. The heptane glue was difficult to use, because when applied to a cover slip, it was either too toxic and killed adult males very quickly or it was too dry to successfully immobilise the flies. Finally, I tested the suitability of cyanoacrylate-based glue, also known as Super Glue, and this was the immobilising tool that worked; flies did not die immediately, nor did they escape. This glue has been used to stick adult flies or pupae in the past, but for a different purpose than to image infection (Pavlidis *et al*., 1994; Schnetzer and Tyler, 1996). To test for toxicity on flies, I tried two different brands of cyanoacrylate-based glue. Flies survived longer when stuck down with Loctite Super Glue instead of Bostik Super Glue Ultra Pen.

When uninfected WT flies survived for at least 24 h while immobilised on a cover slip using the Loctite cyanoacrylate-based glue, I started immobilising and imaging transgenic flies that were expressing enhanced green fluorescent protein (eGFP) or red fluorescent protein (mCherry) in a haemocyte-specific manner. These flies carry *HmlΔGAL4,UAS-2x-eGFP* (lab stocks) or *crq-GAL4,UAS-CD8-Cherry* (a kind gift of C. Wong) transgenes. Since the dorsal abdominal area surrounding the dorsal vessel (in this thesis - the heart region) has a fairly consistent pattern of haemocytes, and has been used as a location of imaging proliferating bacteria in other studies (Akbar *et al*., 2011; Dionne *et al*., 2003), I imaged this area in most instances [Figure 4.1 A and B]. Eventually, I managed to image a single infected fly consecutively for two days [Figure 4.2 A and B, C and D, E and F].

[10] *Live Cell Imaging: A Laboratory Manual*. Subject Area(s): Cell Biology; Microscopy and Imaging; Biotechnology; Laboratory Manuals/Handbooks Edited By Robert D. Goldman, Northwestern University Medical School, Chicago; David L. Spector, Cold Spring Harbor Laboratory, 2005.

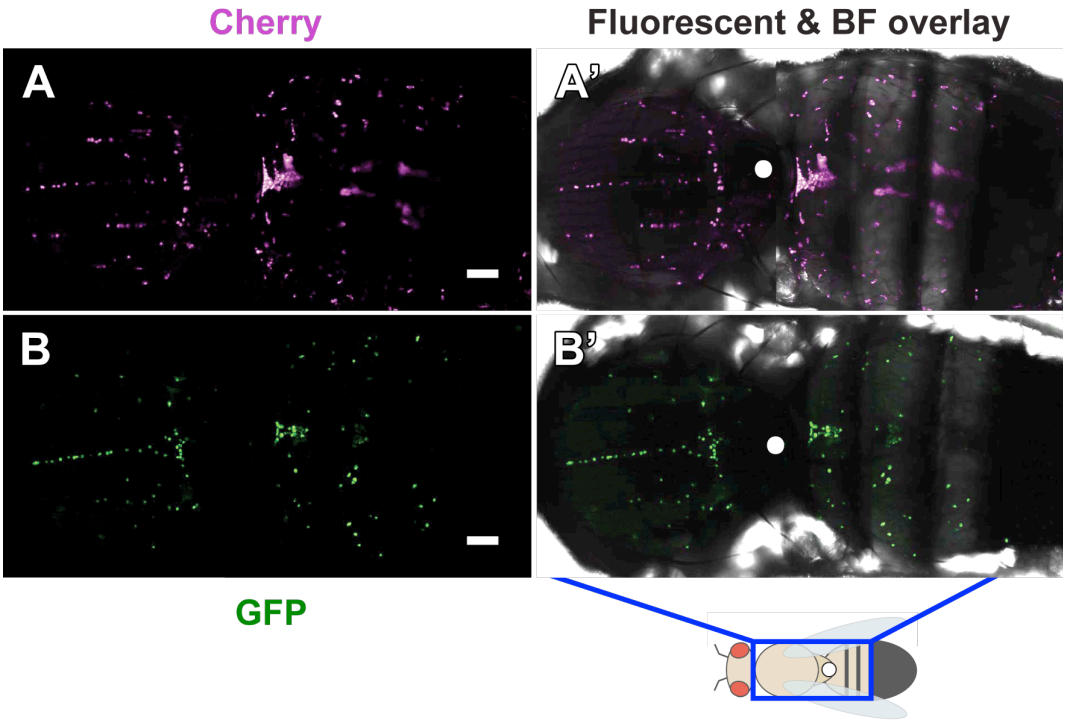


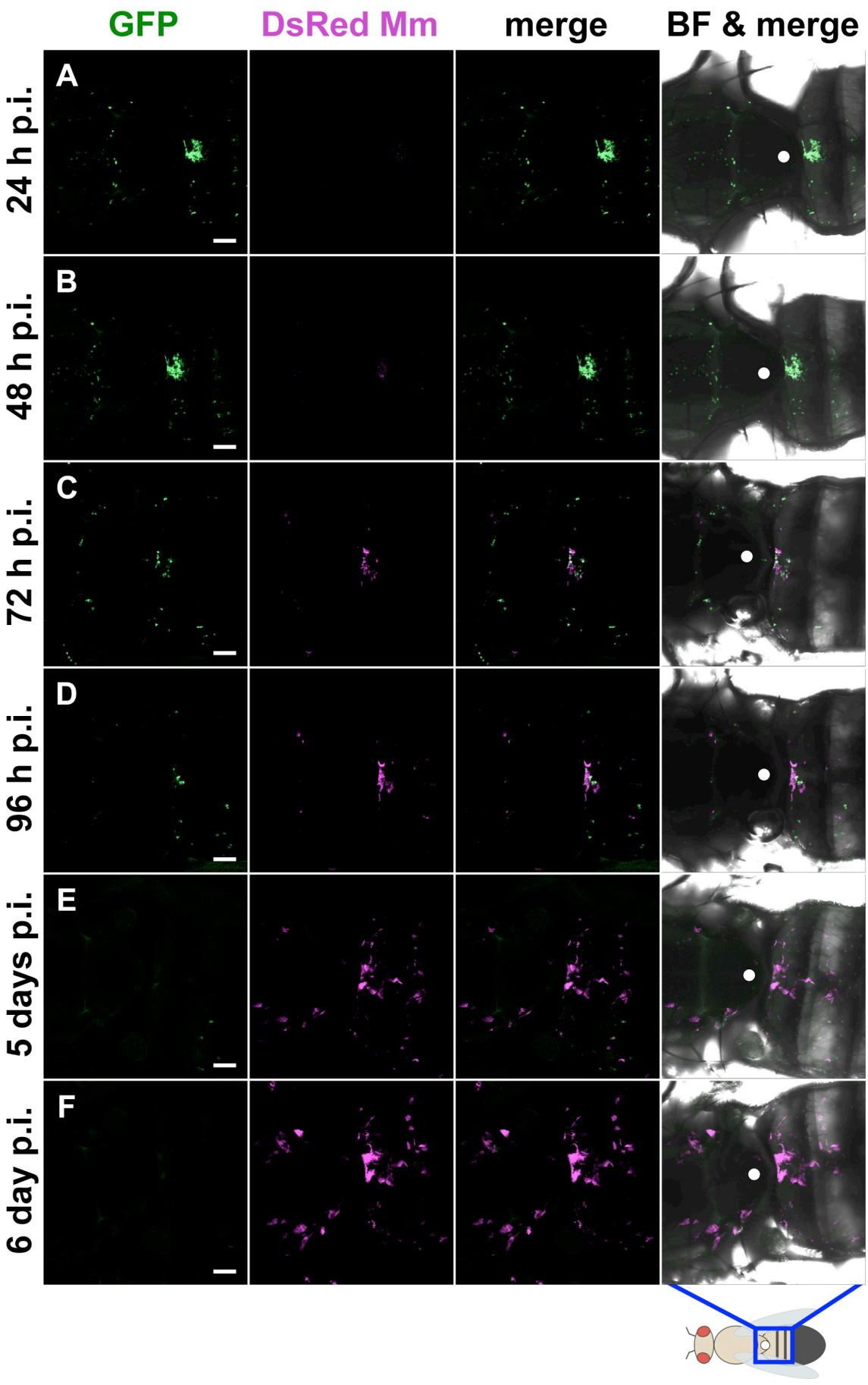
Figure 4.1 **Transgenic flies expressing fluorescent proteins in haemocytes under the control of *crq-GAL4* (magenta) or *HmlΔ-GAL4* (green).** Bacteria or injected pHrodo were imaged in live *Drosophila* males. Haemocytes in the dorsal abdomen and/or thorax of infected or untreated flies were imaged.
A.) Untreated *crq-GAL4,UAS-CD8-Cherry* male fly.
B.) Untreated *HmlΔGAL4,UAS-2x-eGFP* male fly.
Dorsal side of thorax and abdomen was imaged, and is shown here. To ease identification a white dot marks the tip of the fly notum in [A'] and [B']. The dot is also shown in the fly cartoon. The blue rectangle in the cartoon shows the imaged location. Scale bars represent 100 μm.

4.2.2. Progress of infection

Bacterial proliferation in the fly and the subsequent progress of infection was imaged using *M. marinum* expressing fluorescent protein (*DsRed*) under the control of a constitutive promoter *msp* (*msp12::DsRed*) (Clay *et al*., 2007). Unless stated otherwise, *M. marinum* expressing DsRed under the control of *msp12* was used for all infections in this study. Cyanoacrylate-based glue Loctite was used to immobilise transgenic flies expressing eGFP in haemocyte-specific manner. Initially, I injected the same bacterial dose as I would normally use to test survival, 500 CFU. However, at this dose, wild-type flies lived for about 8-9 days and imaging at various time points would prove lengthy. At 72 h p.i., the bacteria were barely visible (data not shown). For the purpose of this study to test *in vivo* imaging of bacterial infection, I used a higher dose, 5000 CFU, of DsRed *M. marinum* to see individual bacteria early and to speed up the infection. Infected flies were imaged at the following time points: 24, 48, 72, 96 h p.i., and in some cases 5 and 6 days post-infection. The survival of flies infected with this high dose was shortened from 8-9 days to 5-6 days (data not shown). An example of an early infection, 24 to 48 h p.i., is shown in [Figure 4.2 A and B]; haemocytes were labelled with eGFP (green), *M. marinum* with DsRed (magenta). Even with the high bacterial dose used, individual bacteria are difficult to find at this early stage of infection. Later on, at 72 h and 96 h p.i., bacteria are clearly visible [Figure 4.2 C and D], and it is apparent that the same fly imaged at two different time points has fewer haemocytes at 96 h p.i. than at 72 h p.i. At later stages of infection, 5 and 6 days p.i., almost no haemocytes are visible, and the bacterial spread is obviously expanded [Figure 4.2 E and F]. The same area of the fly dorsal heart region and part of dorsal thorax is imaged in all cases.

In the majority of cases, when imaging infection, I chose to image the heart region in the dorsal abdomen of the fly. Since the area around the dorsal vessel has a fairly reliable pattern of haemocytes, I hoped to see quite easily a change in phagocytosis or the number of haemocytes or both when imaging the heart region of an *M. marinum*-infected fly. All the initial infection and imaging was done on flies expressing eGFP under the control of *Hml∆*, a haemocyte-specific driver. Later, I used flies expressing mCherry under the control of *crq-GAL4*, also a haemocyte-specific driver.

Figure 4.2 **Progress of *M. marinum* infection from 24 hours to 6 days p.i.**
Drosophilae expressing eGFP (green) in haemocytes were infected with DsRed *M. marinum* (magenta). Each infected fly was imaged at two different time points 24 and 48 h p.i., 72 and 96 h p.i. or 5 and 6 days p.i.
A.) Haemocytes of an infected male #1 at 24 h p.i.
B.) Haemocytes of #1 at 48 p.i.
C.) Haemocytes of #2 at 72 p.i.
D.) Haemocytes of #2 at 96 p.i.
E.) Haemocytes of #3 at 5 days p.i.
F.) Haemocytes of #3 at 6 days p.i.
Scale bars represent 100 μm. White dots in BF & merged panels mark the notum, which is also marked in the fly cartoon. The blue rectangle in the fly cartoon marks the imaged area. At least 4 flies were imaged at each timepoint. *Mm – M. marinum*; BF – bright field.


GFP
DsRed Mm
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A
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24 h p.i.
48 h p.i.
72 h p.i.
96 h p.i.
5 days p.i.
6 day p.i.

4.2.3. High magnification imaging

In order to image individual bacteria and the process of phagocytosis, I imaged haemocytes of DsRed *M. marinum*-infected flies at high magnification. [Figure 4.3] shows haemocytes labelled with eGFP (green) and DsRed *M. marinum* (magenta). The infected fly was imaged at 48 hours p.i.

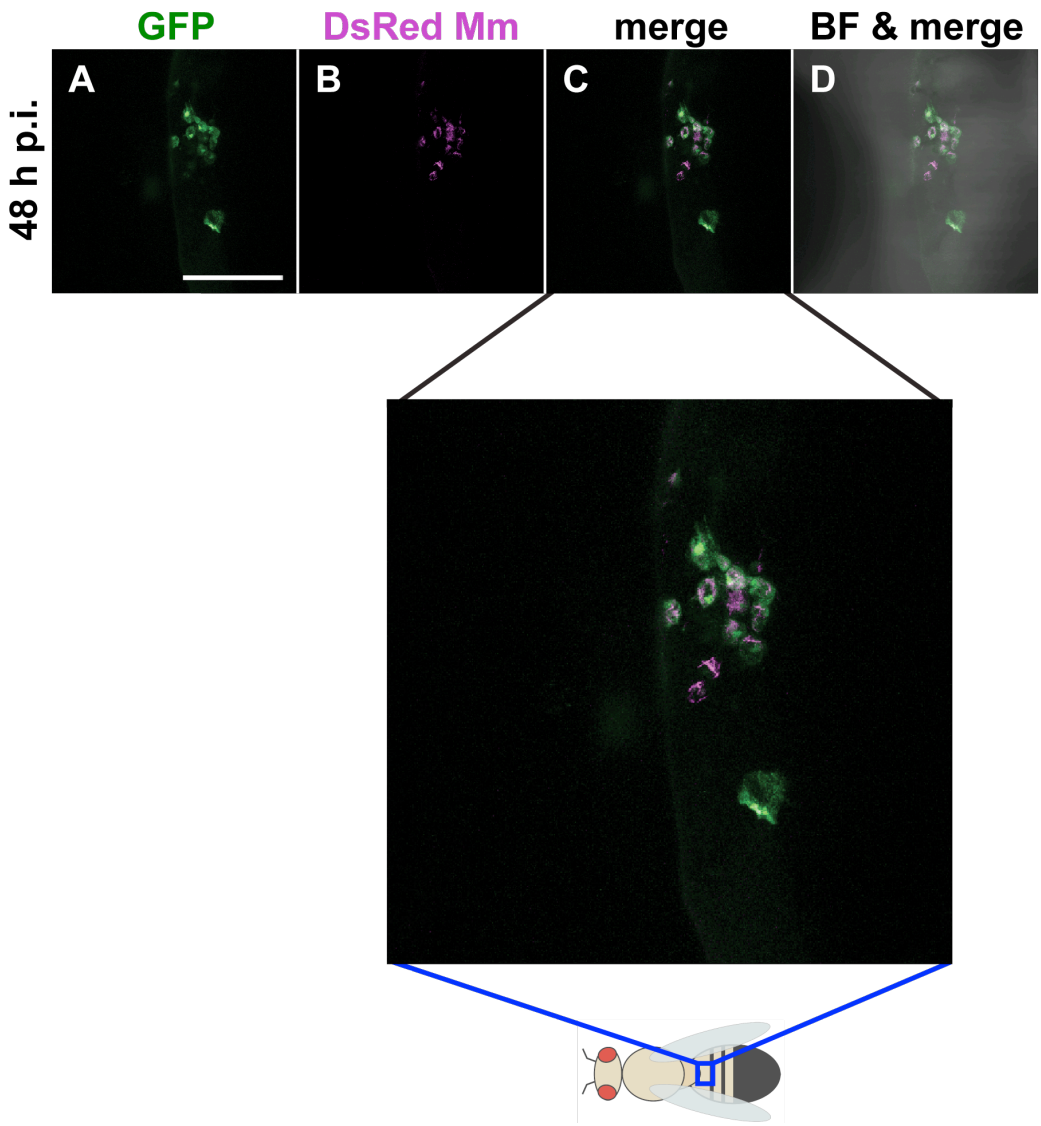


Figure 4.3 **High magnification of infected haemocytes at 48 h p.i.**
A.) Haemocytes (eGFP)
B.) DsRed *M. marinum* (*Mm*; magenta)
C.) *Mm* shown inside haemocytes (merge)
D.) Bright field and merge overlay.
The blue rectangle in the fly cartoon marks the imaged area – the heart region.
Scale bar represents 100 μm.

4.2.4. *M. marinum* infection appears not to induce apoptosis of infected fly macrophages

Drosophila infected with DsRed *M. marinum* repeatedly appeared to lose eGFP-labelled haemocytes with progressing infection. This observation led to various questions:

- "How and why do haemocytes disappear in *M. marinum* infection?"
- "Do the infected cells die of apoptosis?"
- "Do all infected haemocytes die or only a subset that is eGFP^{+ve}?"

In an attempt to answer the first two questions, I used flies overexpressing the anti-apoptotic protein p35 in haemocytes (Hay *et al*., 1994), using *HmlΔ* as a driver, to inhibit apoptotic cell death in haemocytes. First, I infected flies expressing p35 and driver-only controls with *M. marinum*, and tested them for changes in survival time. The standard bacterial dose as used for survival assays, 500 CFU, was administered in this experiment. The survival of *M. marinum*-infected males overexpressing p35 in haemocytes did not differ from that of controls [Figure 4.4].

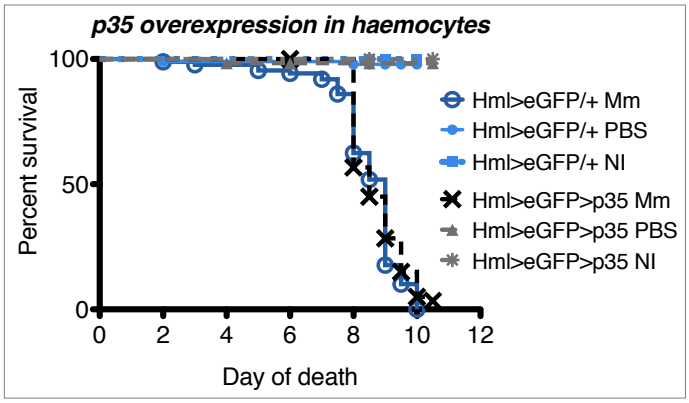


Figure 4.4 **Haemocyte-specific overexpression of the anti-apoptotic protein p35 does not affect survival of *M. marinum*-infected flies.**
The graph is based on four independent experiments, except for the data showing p35 NI controls, which was based on 3 independent experiments; n = min. 47 males. Hml – *HmlΔ; Mm* – *M. marinum*; PBS – mock-infected samples with PBS; NI – untreated.

This transgenic line *UAS-p35* has previously been extensively described and characterised; however, I tested this line by crossing it to the ubiquitous drivers *daughterless* (*da-GAL4*) and *Tubulin* (*Tub-GAL4*) (Bangs and White, 2000; Gaumer *et al*., 2000; Hay *et al*., 1994; Jiang *et al*., 1997; Matthews *et al*., 1989). Since apoptosis is required during metamorphosis for remodelling of larval tissues to form an adult fly, I was expecting to obtain no progeny carrying both the *Tub-GAL4* or *da-GAL4* driver and *UAS-p35* transgene (Bangs and White, 2000; Daish *et al*., 2004; Gaumer *et al*., 2000; White *et al*., 1994). As expected, no progeny carrying both the driver and *UAS-p35* transgene emerged [Figure 4.5].

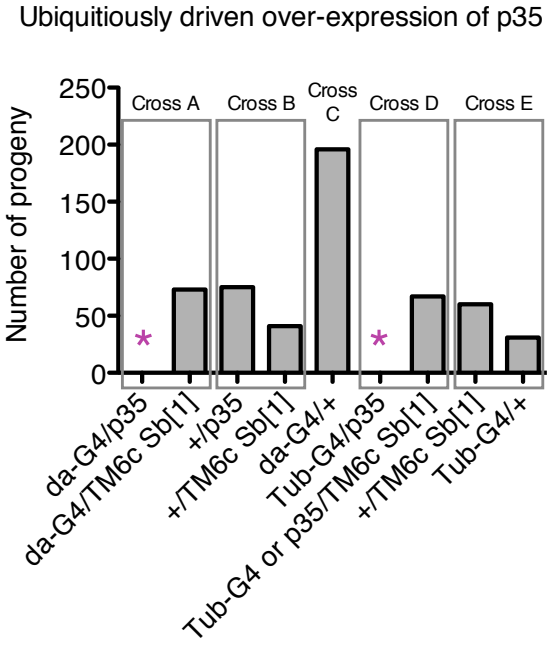
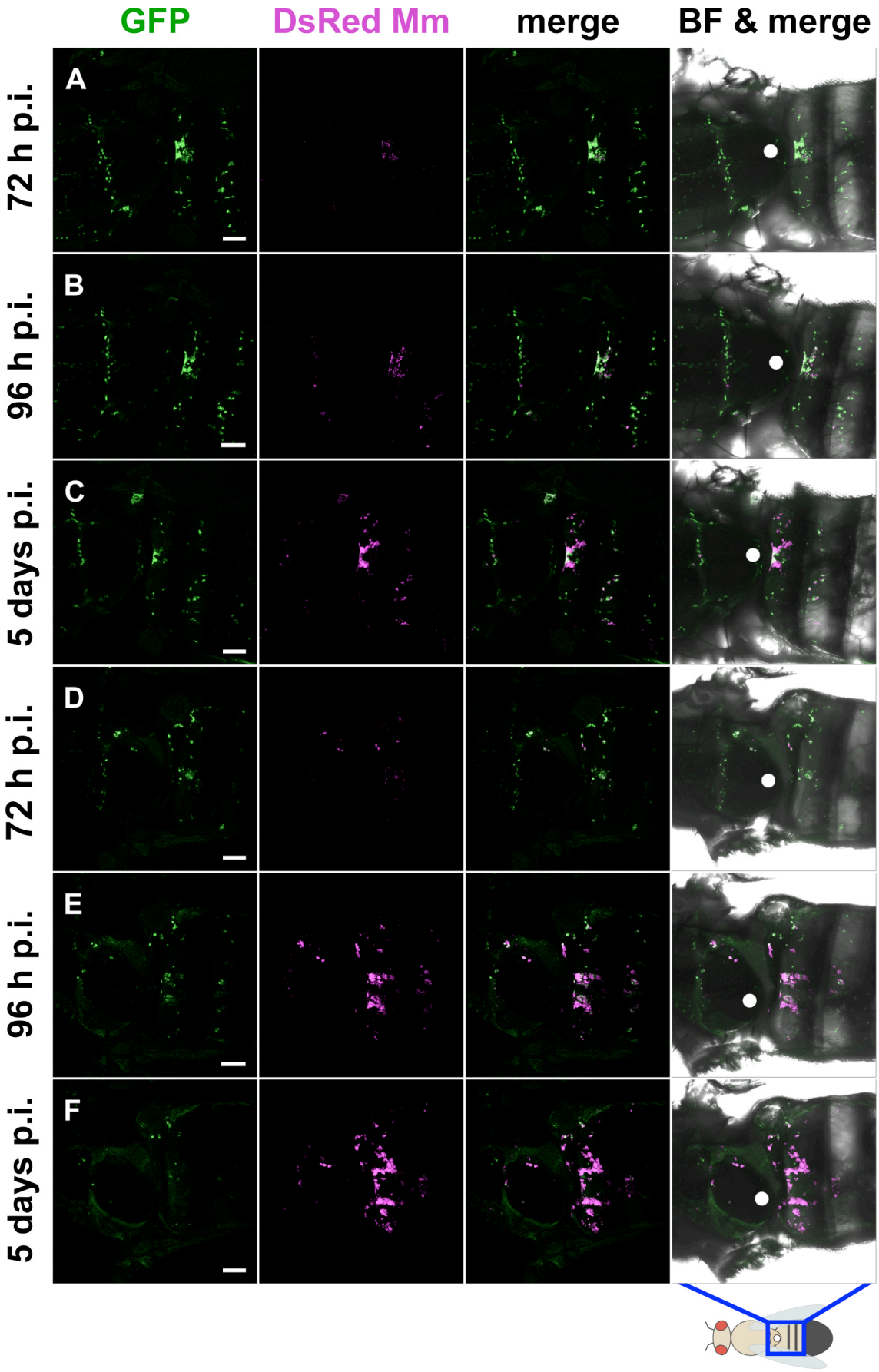


Figure 4.5 ***UAS-p35* works as previously described.**
Inhibition of apoptosis by ubiquitous expression of anti-apoptotic protein p35 gave rise to no progeny. *UAS-p35* line was crossed to flies carrying the ubiquitous driver *Tubulin* or *daughterless*.
Cross A: progeny of *da-GAL4* driver crossed to *UAS-p35*.
Cross B: progeny of WT flies crossed to *UAS-p35* (control).
Cross C: progeny of *da-GAL4* crossed to WT males (control).
Cross D: progeny of *Tub-GAL4* driver crossed to *UAS-p35*.
Cross E: progeny of *Tub-GAL4* crossed to WT males (control).
Pink asterisks show space where no progeny was obtained. More details of crosses A – E are included in the Materials and Methods section.

M. marinum-infected *Drosophila* males overexpressing the anti-apoptotic protein p35 in haemocytes were imaged at different stages of infection [Figure 4.6]. This experiment was done once and the results are contradictory. In one fly [Figure 4.6 A to C], the p35 overexpression seems to have an effect on the number of haemocytes and the growth of bacteria, but in a second infected and imaged fly it appears to be comparable to controls [Figure 4.2 C to E]. This experiment should be repeated to clarify whether one set of the images could be an artefact; however, I was unable to repeat this set of experiments as I was generating fly lines that would allow me to overexpress p35 in adult flies, in temperature-sensitive fashion using *Tub-GAL80* ^{TS}, and *HmlΔGAL4,UAS-2x-eGFP* in the same fly (Lee and Luo, 1999). Additionally, I was planning to use *crq-GAL4*, as a second haemocyte-specific driver (*w*^{1118}*;; crq-GAL4, UAS-2x-eGFP*), to test p35 overexpression in haemocytes in temperature-sensitive fashion, and subsequently a difference in survival in *M. marinum* infection.

To answer the third question: "Do all infected haemocytes die or only a subset that is eGFP-positive?", I obtained *crq* RNAi line (*UAS-crq.IR*) to knock down the expression of *crq* in haemocytes using *HmlΔ* as a driver. This would be done in flies expressing *GAL4*-independent haemocyte-specific nuclear DsRed (*w;HmlΔDdsRed.nuc*) using *Tub-GAL80* ^{TS} to avoid developmental effects of knocking down the expression of *croquemort* (Clark *et al*., 2011; Lee and Luo, 1999). Due to insufficient time, I was unable to complete this experiment; however, from a study by Clark *et al*., 2011 it is clear that subsets of haemocytes with differential expression of haemocyte-specific markers indeed exist in adult flies (Clark *et al*., 2011).

Figure 4.6 **Progress of *M. marinum* infection in *Drosophila* overexpressing the anti-apoptotic protein p35 in haemocytes.** Flies were infected with DsRed *M. marinum* at the usual concentration of approximately 5000 CFU per fly. Two different infected flies are shown; each was imaged at three different time points 72 and 96 h p.i., and 5 days p.i. Haemocytes were labelled with GFP (green), and *M. marinum* expressing *DsRed* under the constitutive promoter *msp12* (Clay et al., 2007).
A.) and **D.)** Haemocytes of infected males at 72 h p.i.
B.) and **E.)** Haemocytes of infected males at 96 h p.i.
C.) and **F.)** Haemocytes of infected males at 5 days p.i.
The first infected fly [A] – [C] appears to have more haemocytes and a fewer bacteria when compared to controls [Figure 4.2 C - E]. However, the second infected fly is comparable to controls. Scale bars represent 100 μm. White dots in BF & merged panels and in the fly cartoon mark the notum. The blue rectangle in the fly cartoon marks the imaged area. *Mm* – *M. marinum*; BF – bright field.


GFP
DsRed Mm
merge
BF & merge
A
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D
E
F
72 h p.i.
96 h p.i.
5 days p.i.
72 h p.i.
96 h p.i.
5 days p.i.

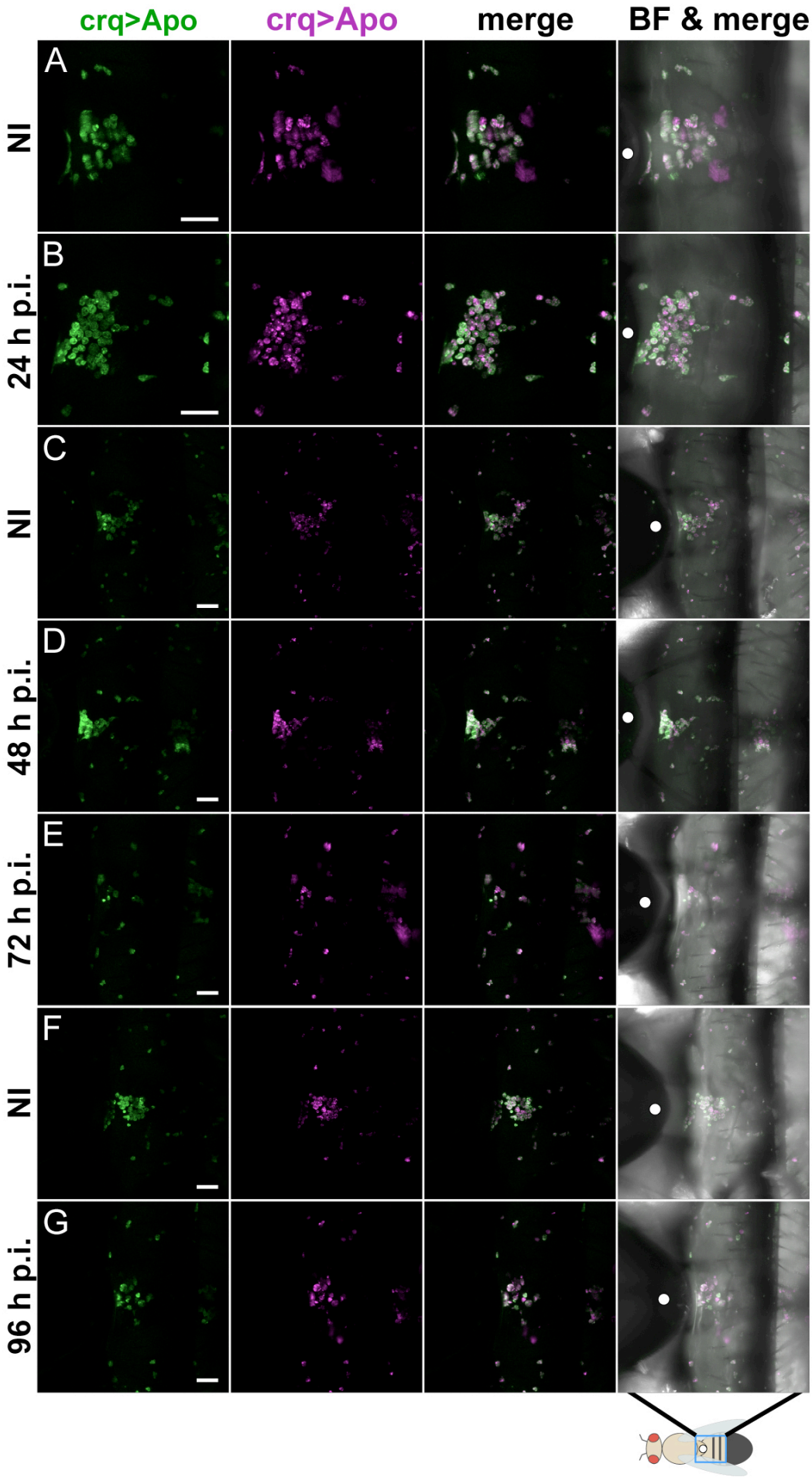
4.2.5. Apoliner

Apoliner is a reporter of caspase activity (Bardet *et al*., 2008). The rationale behind testing this caspase activity sensor was to see if *M. marinum*-infected haemocytes undergo apoptosis. Apoliner is a sensor consisting of: 1. a transmembrane domain; 2. mRFP; 3. a caspase substrate BIR (Baculovirus Inhibitor of Apoptosis Repeat); 4. nuclear localisation signal; and 5. eGFP (Bardet *et al*., 2008). Under normal conditions both fluorophores are localised at the cell membrane. If a cell undergoes apoptosis and caspases are active, the BIR domain is cleaved, thus separating the fluorophores. mRFP would remain at the cell membrane and eGFP, fused to the nuclear localisation signal, would translocate to the nucleus (Bardet *et al*., 2008).

D. melanogaster was crossed so as to obtain a progeny expressing the Apoliner transgene in a haemocyte-specific manner, under the control of the *HmlΔ* or *crq* promoter. I established that the Apoliner transgene was expressed well only in one of three lines, in a line carrying the transgene on the third chromosome. Also, *crq* proved to be a stronger driver, and was used to drive the Apoliner transgene in subsequent experiments [Figure 4.7] No haemocyte apoptosis was detectable upon infection with *M. marinum* [Fig 1.7] or with the *imd* agonist bacteria *E. coli* (5 h p.i.) and *Enterobacter cloacae* (24 h p.i.) [data not shown]. The images in [Figure 4.7] show a maximised projection of a stack of images 80 – 90 μm deep. The starting point of every stack was determined by focusing on a particular part of the dorsal surface of the abdomen of each fly, so as to ensure a consistent proportion imaged of each fly. The end of the stack was marked 80 – 90 μm into the fly, depending on whether haemocytes were still visible at this depth. Since this covers the visible depth in the fly in my experiments, and this experiment was done before the period of visible haemocyte disappearance, these data suggest that haemocytes are not simply being lost from the focal plane during infection,

and that haemocytes are not dying via caspase-dependent apoptosis. It does not distinguish, however, between non-caspase-dependent forms of cell death, including autophagy or necrosis (Deretic and Levine, 2009; Shelly *et al*., 2009; Trump *et al*., 1997; Yano *et al*., 2008).

Figure 4.7 **Haemocytes of *M. marinum*-infected flies appear not to undergo apoptosis.** Haemocytes are made visible using the haemocyte-specific driver *crq-GAL4*. The fluorescent proteins are expressed in haemocytes due to the presence of the Apoliner transgene. eGFP portion of Apoliner expressed specifically in haemocytes is shown in green. mRFP portion of Apoliner expressed specifically in haemocytes is shown in magenta. eGFP and mRFP co-localisation is shown as a merge, and bright field and fluorescent channels (BF & merge) overlay is shown in the right-most panels. At least 4 flies were imaged per genotype, per condition. Each confocal image represents flattened maximised projection of 80 -90 μm.
A.) Respective NI controls to [B].
B.) Apoliner eGFP and mRFP localisation at 24 h after *Mm* infection.
C.) Respective NI controls to [D].
D.) Apoliner eGFP and mRFP localisation at 48 h after *Mm* infection.
E.) Apoliner eGFP and mRFP localisation at 72 h after *Mm* infection.
F.) Respective NI controls to [G].
G.) Apoliner eGFP and mRFP localisation at 96 h after *Mm* infection (green).
Scale bars represent 100 μm. White dots in the [BF & merge] panels mark the notum, which is also marked in the fly cartoon. The blue rectangle in the fly cartoon marks the imaged area. *Mm* – *M. marinum*; NI - untreated; BF – bright field; *crq* - *crq-GAL4*.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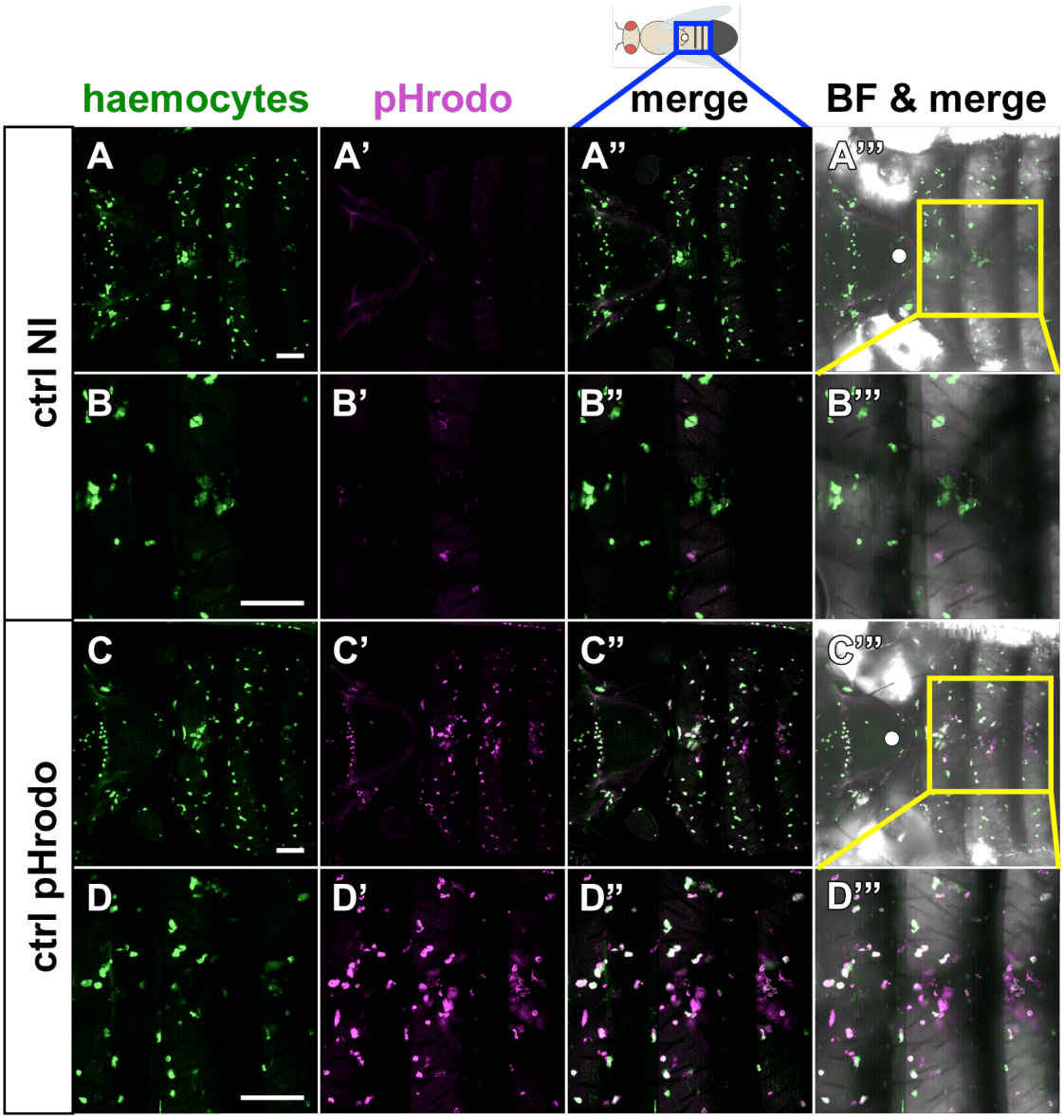
NI
G
96 h p.i.

4.2.6. Imaging of pHrodo-labelled *E. coli in vivo*

Phagocytosis can be imaged using non-infectious polystyrene, latex or other microbeads (Akbar *et al*., 2011; Cuttell *et al*., 2008; Elrod-Erickson *et al*., 2000). pHrodo-labelled *E. coli* BioParticles® (pHrodo) is a rhodamine-based dye that is conjugated to dead bacteria and used as a probe for phagocytosis. At a low pH, e.g. in phagocytic vesicles, it becomes fluorescent red. pHrodo was used *in vivo* in adult flies in other studies (Akbar *et al*., 2011; Cuttell *et al*., 2008).

pHrodo was used as a test to determine the function of haemocytes in otherwise untreated flies [Figure 4.8 C and D]. Controls were untreated and imaged alongside pHrodo-injected flies [Figure 4.8 A and B]; some red autofluorescence is visible, but in comparison to pHrodo-injected flies it is negligible. The obtained images suggest that the overlap of eGFP expressing cells and that of pHrodo present in acidified vesicles is good, but not complete [Figure 4.8 D" and D'"]. In the this figure, some cells contain pHrodo and are GFP^{+ve}, some, on the other hand, are only $pHrodo^{+ve}$ or GFP^{+ve}. It has been recently shown that subsets of haemocytes exist and express different markers (Clark *et al*., 2011). It is possible that [Figure 4.8] does not show other phagocytic cells, but shows haemocytes that engulfed pHrodo dye, but did not express *Hemolectin*.

Figure 4.8 ***Hemolectin*** **appears to drive eGFP expression in many, but not all haemocytes.** *Hml>eGFP* males were imaged untreated or injected with pHrodo. pHrodo was injected to test how well it co-localises with $eGFP^{+ve}$ haemocytes (green). Flies were imaged within 2 hours of pHrodo injection. The pHrodo particles (magenta) are localised to haemocytes. This pattern is consistent with the pattern of untreated controls (NI) that express eGFP in a haemocyte-specific manner [A].
A.) Haemocytes of NI males; **A'.)** Phagocytosed pHrodo; **A".)** Haemocytes & pHrodo co-localisation (merge); **A'".)** Bright field and fluorescent channels (BF & merge) overlay.
B.) Close-up of area in [A] in the yellow rectangle.
C.) Haemocytes of pHrodo-injected males; **C'.)** Phagocytosed pHrodo; **C".)** Merge; **C'".)** BF & merge
D.) Close-up of area in [C] in the yellow rectangle
At least 3 flies were imaged per each condition. Scale bars represent 100 μm. The cartoon shows the dorsal side of *D. melanogaster*; the blue rectangle marks the area that was imaged; white dots in the cartoon, [A'"] and [C'"] mark the notum.


haemocytes
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D'''

4.3. Conclusion

The aim of this study was to establish techniques for imaging *M. marinum* infection *in vivo*. The use of a cyanoacrylate-based glue to immobilise infected flies and controls makes it possible to image progress of infection in the same fly at two or three time points, 24 hours apart. Keeping immobilised flies alive for 24 hours requires patience and careful manipulation, but is not complicated.

Imaging DsRed *M. marinum*-infected flies provided solid results that eGFP-positive cells disappeared with progressive infection when eGFP was driven with *Hemolectin*. Results from the tests using pHrodo suggest that not all haemocytes are Hml^{+ve}: co-localisation of pHrodo and eGFP-labelled haemocytes was not 100%. It is not yet clear whether all haemocytes, or just the Hml^{+ve} population, disappear during *M. marinum* infection.

This method of immobilising and imaging requires improvement in timing so that the process of phagocytosis of bacteria is imaged. However, I believe that this method offers several advantages:

1. is faster and cheaper than that of antibody staining of haemocytes with (Seroude *et al*., 2002);
2. allows imaging of progressive changes (time lapse) *in vivo* [Figure 4.2];
3. offers reliable *in vivo* imaging that requires minimal sample manipulation (fixing, embedding, sectioning etc.).

Also, this imaging method has been successfully utilised by my colleagues (Clark *et al*., 2011).

Haemocyte-specific overexpression of anti-apoptotic protein p35 does not affect the survival of *D. melanogaster* during *M. marinum* infection. However, the expression

of *UAS-p35* transgene was driven using *HmlΔ-GAL4*. This set-up did not allow for precise time-specific overexpression of the anti-apoptotic protein p35, and thus could have affected the development of haemocytes. In order to achieve greater precision and overexpress p35 in adults, I intended to use *Tub-GAL80^{TS}* to eliminate developmental effect of p35 overexpression. The main reasons why this set of experiments, using haemocyte-specific p35 overexpression, was not repeated were not to waste time and resources on a procedure that was not sufficiently informative, and to generate more suitable fly lines for this purpose. Due to time constraints, I did not manage to finish generating the fly lines and to repeat this set of experiments – imaging and survival.

The results using Apoliner transgene expressed under the control of the *crq-GAL4* suggest that haemocyte death after *M. marinum* infection is not associated with increased caspase activity. Future work will characterize positive host factors in this death and help resolve what caspase-independent mechanisms are at work.

Chapter 5. FOCUSED SCREEN OF *DROSOPHILA* GENES INVOLVED IN THE PHAGOCYTOSIS TO REVEAL *M. MARINUM*-SPECIFIC PHAGOCYTIC RECEPTORS

Abstract

Phagocytosis is an important immune process that is altered by many bacteria that are successful human pathogens. *M. tuberculosis* is one such pathogen; it is closely related to *M. marinum*, which does not cause systemic disease in people. This screen was an attempt to reveal *Mycobacterium marinum*-specific phagocytic receptors of the fruit fly *Drosophila melanogaster*. The adult *Drosophila* phagocytic cell is the plasmatocyte and is the fly equivalent of the vertebrate macrophage. RNAi lines targeting the various known phagocytic and scavenger receptors were used to silence these genes in a plasmatocyte-specific manner, using a *Hemolectin* promoter (*Hml*Δ) as a driver. Knockdown flies were then tested for survival and bacterial load after *M. marinum* infection and analyzed by microscopy.

Plasmatocyte-specific knockdown of the *nimrod C3* (*nimC3*) gene resulted in a significantly decreased *M. marinum* load, and it blocked expression of antimicrobial peptide Metchnikowin in *M. marinum*-infected flies. Nimrod C3 is a phagocytic receptor that belongs to the Nimrod family, as does Nimrod C1 – a receptor necessary for phagocytosis of *S. aureus* (Kurucz *et al.*, 2007). This phenotype was not associated with a clear change in phagocytic activity against *M. marinum*, implying that the knockdown may alter some other aspect of phagocyte function.

5.1. Introduction

5.1.1. Phagocytosis

Phagocytosis is a process during which pathogens, apoptotic cells or debris, and even synthetic foreign objects (e.g. latex beads) are engulfed and destroyed or recycled within a phagocytic cell. The mammalian macrophage is an example of a phagocytic cell. The macrophage equivalent in *D. melanogaster* is the plasmatocyte (Franc *et al*., 1999a; Rizki and Rizki, 1980). In order to recognise the appropriate 'material' for phagocytosis, recognition receptors are expressed on the cell surface of a phagocytic cell. Following the detection of a pathogen (or apoptotic corpse), surface molecules of this pathogen, such as bacterial LPS, are recognised and bound by phagocytic/scavenger receptors on the surface of a macrophage or plasmatocyte (Rämet *et al*., 2001; Stuart and Ezekowitz, 2005). The phagocytic cell undergoes cytoskeletal changes; the phagocytosing 'leading edge' accumulates filamentous actin (F-actin) and other proteins. The activation of the Rho GTPase signalling pathway triggers a rearrangement of actin cytoskeleton, and so allows the engulfing of pathogens or apoptotic debris (Greenberg and Grinstein, 2002). The phagocytosed 'material' becomes compartmentalised within a vacuole – phagosome – that undergoes the process of maturation. During phagosome maturation, various proteins, such as vacuolar ATPase (V-ATPase) and acidic proteases are trafficked to the phagosome (Greenberg and Grinstein, 2002). The maturation progresses by the fusion of the phagosome with acidic lysosomes, thus forming phagolysosome. V-ATPase reduces pH, which in turn activates acidic proteases that are important in the final stages of phagosomal deactivation or recycling (Kinchen and Ravichandran, 2008; Stuart and Ezekowitz, 2005). The terms plasmatocyte and haemocyte (the fly 'blood cell') are used interchangeably here.

5.1.2. *Mycobacterium tuberculosis* and *Mycobacterium marinum*

Some pathogens can interfere with the normal process of phagocytosis for their own benefit; *Francisella tularensis* is capable of escaping from the phagosome (Barker *et al*., 2009); *M. tuberculosis* and *Legionella pneumophila* are examples of pathogens capable of 'hijacking' the process of phagosome maturation by preventing the phagosome-lysosomal fusion (Clemens and Horwitz, 1995; van der Wel *et al*., 2007; Vergne *et al*., 2004).

M. tuberculosis pathogenesis and its subversion of phagosome maturation has been studied (Vergne *et al*., 2004). However, *M. tuberculosis* is difficult and expensive to study because any work with this pathogen requires a Biosafety Level 3 (BSL-3) working environment. Also, a single tuberculosis experiment in a wild-type mouse takes more than 80 days to complete (Hölscher *et al*., 2008), thus increasing the cost substantially. *M. tuberculosis'* close relative, *M. marinum*, does not cause systemic disease in people and does not require BSL-3 containment, and so it is useful in modelling some aspects of *M. tuberculosis* pathogenesis (Tønjum *et al*., 1998). *M. marinum* has been used as a pathogen in various non-mammalian models - *Dictyostelium discoideum* (slime mould), *Danio rerio* (zebrafish) and *Drosophila melanogaster* (fruit fly) (Clay *et al*., 2007; Dionne *et al*., 2003; Hagedorn and Soldati, 2007). The goal of this project was to perform a preliminary screen to identify *Drosophila* phagocytic receptors that could be specific for the uptake of *M. marinum* and the subsequent infection by this bacterium. *M. marinum* was used throughout this work to perform survival assays, and to analyse the bacterial load and levels of AMP expression in infected flies. The screen was performed using *Drosophila* RNA interference (RNAi) lines targeting various phagocytic receptors in a plasmatocyte-

specific manner.

5.1.3. Phagocytic receptors

The main phagocytic cell type of *Drosophila* is the plasmatocyte, which is functionally equivalent to the vertebrate macrophage (Rämet *et al*., 2001). Phagocytosis is an important process in normal development and immune defence (Defaye *et al*., 2009; Franc *et al*., 1999b); however, I will focus on the immune side of phagocytosis and the genes coding for phagocytic or scavenger receptors of *D. melanogaster*.

The *Drosophila* phagocytic and scavenger receptors are discussed below, and are grouped according to their classification – the CD36 scavenger receptor family, the *Drosophila* scavenger receptor class C, and miscellaneous phagocytic receptors, including the Nimrod family. Many members of the *Drosophila* phagocytic families are not well known, or their function in respect of infection and immunity is not known. A more detailed account of the other *Drosophila* phagocytic receptors is included in chapter 1 (General introduction). This results chapter discusses only the phagocytic receptors for which the fly RNAi lines were available, and were used in this screen to reveal a *M. marinum*-specific phagocytic receptor.

5.1.3.1. Cluster of Differentiation 36 (CD36) scavenger receptor family

The genes that belong to this family of *Drosophila* receptors share a homology with the human class B scavenger receptor cluster of differentiation 36 (CD36), a membrane protein that plays a role in apoptosis (Savill *et al*., 1992; Stuart *et al*., 2005). The *Drosophila croquemort* gene is required for apoptosis and the phagocytosis of *S. aureus* (Franc *et al*., 1996; Franc *et al*., 1999a; Stuart *et al*., 2005); however, the *crq* gene was not included in this screen, because no RNAi line was available at the time. Another fly member of this family is the scavenger receptor **Peste** (Pes) that has so far been the only

fly receptor described to be mycobacterium-specific. RNAi silencing of the *peste* gene *in vitro* leads to a reduced phagocytosis of *Mycobacterium fortuitum* and *M. smegmatis*, but the uptake of other bacteria, such as *E. coli* and *S. aureus*, is not affected (Philips *et al*., 2005).

5.1.3.2. *D. melanogaster* class C scavenger receptor

Out of the *Drosophila* scavenger receptor class C, the class I (**SR-CI**) is known to play a role in *D. melanogaster* immunity; it is required for the phagocytosis of Gram-positive and Gram-negative bacteria *in vitro* (Pearson and Lux, 1995; Rämet *et al*., 2001). Other members of this scavenger receptor family are not well characterised in relation to infection. However, polymorphic markers in the loci of the fly *Sr-CI - Sr-CIV* genes are variably associated with the resistance to naturally occurring bacteria, Gram-negative bacteria (*Serratia marcescens, Providencia burhodogranaria*) and Gram-positive (*Lactococcus lactis*, *Enterococcus faecalis*) (Lazzaro *et al*., 2006). The scavenger receptors included in this screen were SR-CI, SR-CII, and SR-CIV.

5.1.3.3. Miscellaneous *D. melanogaster* phagocytic receptors

The Nimrod family members were included in this screen (Kocks *et al*., 2005); however, only one member, **Nimrod C1** (NimC1), has been previously connected to phagocytosis of bacteria. The *nimC1* gene knockdown *in vitro* resulted in reduced phagocytosis of *S. aureus*; overexpression, on the other hand, led to increased phagocytosis of *S. aureus* and *E. coli* (Kurucz *et al*., 2007). Similar to the Nimrod family, the ***eater*** gene has been shown to be important in immunity. The knockdown of **Eater** using RNAi *in vitro* resulted in reduced phagocytosis of bacteria, and was found to bind live or dead Gram-positive bacteria (Chung and Kocks, 2011; Kocks *et al*.,

2005). **Down syndrome cell adhesion molecule** (Dscam) was shown to be involved in phagocytosis of bacteria in *D. melanogaster*. Loss of Dscam in haemocytes impaired their phagocytic function; the uptake of heat-killed *E. coli* was significantly reduced (Watson *et al*., 2005).

Based on a small-scale screen of *Drosophila* phagocytic receptors, the *nimrod C3* gene appeared to have a phenotype in connection with *M. marinum* infection. As a result, it was investigated further with the prospect of identifying *M. marinum*-specific receptor in *Drosophila*.

5.1.3.4. Nimrod C3

Nimrod C3 (NimC3) belongs to a family of transmembrane proteins that are expressed on *D. melanogaster* plasmatocytes, the fly equivalent of vertebrate macrophages (Kurucz *et al*., 2007; Rämet *et al*., 2001). NimC3 protein has not been well studied, but the function of other members of the Nimrod family has been described. NimC1 is required for the phagocytosis of bacteria *in vitro*, and NimC4 is a phagocytic receptor necessary for the recognition of apoptotic cells in the developing *Drosophila* embryo (Kurant *et al*., 2008; Kurucz *et al*., 2007). Apart from the genes *nimC1* and *nimC4*, the function of the *nimrod* genes is not known.

5.1.4. Summary

Phagocytosis is an essential process in maintaining homeostasis and preventing disease; however, some pathogens are capable of 'cheating' phagocytes and using them to be shielded from the immune system and to grow (Barker *et al*., 2009; Kinchen and Ravichandran, 2008; Stuart and Ezekowitz, 2005). Pathogenic mycobacteria, such as *M. tuberculosis*, can prevent phagosome maturation, and successfully replicate within the host, including humans (Barker *et al*., 2009; Kinchen and Ravichandran, 2008; Stuart and Ezekowitz, 2005). *M. marinum* is closely related to *M. tuberculosis,* and *D. melanogaster* is a proven model for studying various infections, including that caused by *M. marinum* (Dionne *et al*., 2003; Tønjum *et al*., 1998).

The aim of this screen was to identify a *M. marinum*-specific phagocytic receptor in the fly with RNAi targeting genes involved in phagocytosis using the *Hemolectin* promoter to achieve a haemocyte-specific silencing. However, not all known *Drosophila* scavenger or phagocytic genes were tested in this screen. The role of the various phagocytic receptors was tested on several levels – by assessing the survival of *M. marinum*-infected flies, by quantifying the bacterial load of *M. marinum* using qRT-PCR and *M. marinum* specific primers, and by *in vivo* imaging of selected RNAi lines to find a potential phagocytosis phenotype in haemocyte-specific knockdown.

In this work I tested the role of phagocytic receptors in isolation in respect to *M. marinum* specificity. It is very likely that more than one receptor is required for the detection and engulfment of *M. marinum in vivo*. In most cases, successful uptake of bacteria or apoptotic cells requires the phagocytic receptor to interact with other proteins, for example the CD36 scavenger receptor interacts with vitronectin in mammalian macrophages in order to phagocytose apoptotic neutrophils (Savill *et al*.,

1992). However, some proteins may be redundant and in this case a loss-of-function screen of single candidates would not uncover *M. marinum*–specific receptor.

5.2. Results

5.2.1. Haemocyte-specific drivers

At the time of doing this work, *Hemolectin* was a convincing haemocyte-specific driver in the adult *Drosophila* (Defaye *et al*., 2009); however, at least one more haemocyte-specific driver was desirable. Therefore, I tested the other genes that are haemocyte-specific - *croquemort* (*crq*) and *Hemese* (*He*) (Franc *et al*., 1996; Zettervall *et al*., 2004). The *crq* and *He* promoters were tested as drivers for the expression of the following fluorescent proteins: a myristoylated membrane monomeric red fluorescent protein (myr-mRFP), nuclear Discosoma sp. Red fluorescent protein (DsRed), enhanced yellow fluorescent protein (eYFP), and enhanced green fluorescent protein (eGFP). The test rendered a single driver-fluorescent protein combination (*w[1118]; UAS-2xeYFP/crq-GAL4*) that produced a visible, but weak fluorescent signal in the adult *Drosophila* [Table 5.1]. In the subsequent experiments, I used *HmlΔ* as the sole haemocyte-specific driver.

Cross		**Progeny**
Virgins	**Males**	**Fluorescent**
w[1118]; UAS-myr-mRFP/SM6a	*crq-GAL4*	*No*
w[1118];; UAS-myr-mRFP/TM6c, Sb[1]	*He-GAL4*	*No*
w; UAS-DsRed2.Nuc21/CyO;	*crq-GAL4*	*No*
w;; UAS-DsRed2.Nuc22	*He-GAL4*	*No*
w[1118]; UAS-2xeYFP/SM6a;	*crq-GAL4*	*Weak*
w[1118];; UAS-2xeYFP/TM6c, Sb[1]	*He-GAL4*	*No*
yw; UAS-2xeGFP;	*crq-GAL4*	*No*

Table 5.1 **Haemocyte-specific drivers – *croquemort* (*crq-GAL4*) and *Hemese* (*He-GAL4*).** Several fluorescent proteins were tested under the control of *crq* or *He*. The initial testing of these haemocyte-specific drivers was successful in one case only – *crq > 2xeYFP*. The eYFP expression was weak; therefore *HmlΔ* was used as the only haemocyte-specific driver in this study.

5.2.2. CD36 scavenger receptor family

Drosophila members of the class B CD36 scavenger receptor family were used to test for differences in survival after *M. marinum* infection. In the selected RNAi lines, bacterial load was quantified using qRT-PCR, and some members were included in the *in vivo* imaging screen.

D. melanogaster **CD36 scavenger receptor genes (Nichols and Vogt, 2008)**		
Gene name	**Gene abbreviation**	**Gene ID**
peste	*pes*	*CG7228*
-	-	*CG1887*
Sensory neuron membrane protein 1	*Snmp1*	*CG7000*
Sensory neuron membrane protein 2	*Snmp2*	*CG7422*
-	-	*CG10345*
-	-	*CG7227*
-	-	*CG2736*
-	-	*CG3829*

Table 5.2 ***D. melanogaster*** **genes coding for the CD36 scavenger receptors.** *Drosophila* RNAi lines targeting these genes were used in this screen in an attempt to reveal a *M. marinum*-specific phagocytic receptor in the fly.

To examine the effect of haemocyte-specific silencing of various phagocytic genes on the progress of *M. marinum* infection of *Drosophila*, I crossed the transgenic flies carrying *HmlΔGAL4,UAS-2xeGFP* to those carrying *UAS-gene-of-interest.IR*. The driver only control flies were heterozygous for the *HmlΔGAL4,UAS-2xeGFP* transgene. To test if survival of *M. marinum*-infected control flies was comparable to the WT *D. melanogaster*, I used the w^{1118} (DrosDel isogenic background). eGFP under the control of *Hemolectin* promoter was used to image the pattern of haemocyte distribution in the dorsal abdomen and/or thorax of the control and knockdown flies.

5.2.2.1. Survival

The survival of a driver-only control, *w[1118]; HmlΔGAL4,UAS-2xeGFP*/+, was compared to WT *w[1118]* males (DrosDel isogenic background). The difference between the survival curves was not statistically significant [Figure 5.7 A]. The results of survival assays show that haemocyte-specific knockdown of *Snmp1*, *CG3829*, *CG1887*, *CG2736*, *CG10345*, or *Snmp2* results in significantly increased survival of *M. marinum*-infected flies [Figure 5.1 A - G].

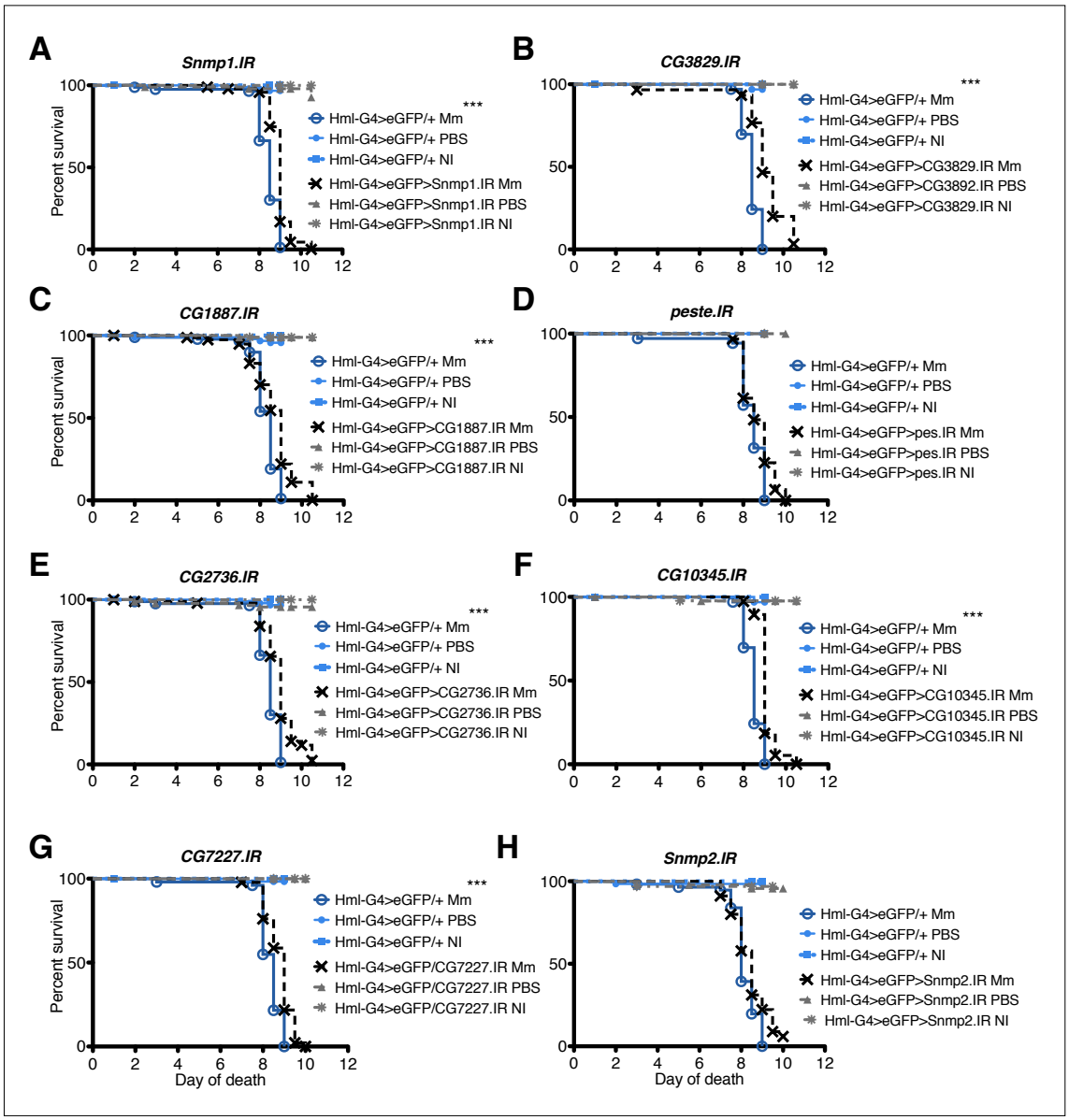


Figure 5.1 **Survival of *D. melanogaster* with haemocyte-specific knockdown of genes belonging to the CD36 scavenger receptors after *M. marinum* infection.**
Results are shown for only a subset of the tested knockdowns. The fruit flies were infected with 500 CFU of WT *M. marinum*.
A.) *Snmp1* knockdown, the result is based on 5 ind. exp., n = min. 83 *Drosophila* males per genotype per condition.
B.) *CG3829* knockdown (2 ind. exp.), n = min. 27.
C.) *CG1887* knockdown (5 ind. exp.), n = min. 78.
D.) *peste* knockdown (2 ind. exp.), n = min. 31.
E.) *CG2736* knockdown (5 ind. exp.), n = min. 81.
F.) *CG10345* knockdown (2 ind. exp.), n = min. 27.
G.) *CG7227* knockdown (3 ind. exp.), n = min. 40.
H.) *Snmp2* knockdown (3 ind. exp.), n = min. 45; however, the data of NI controls of *Snmp2* knockdown is based on 2 ind. exp. only, n = 32.
The statistical significance between survival curves was determined using Log-rank analysis (Mantel-Cox, GraphPad Prism); *** $p < 0.0001$. *Mm – M. marinum*, PBS – PBS-injected controls, NI – untreated controls, ind. exp. - independent experiments.

5.2.2.2. Bacterial load

The RNAi lines for *CG1887*, *Snmp1*, *CG2736*, and *CG7227* were tested for differences in *M. marinum* load 6 days after infection. *Snmp1* knockdown line was used in a second experiment because the first result had given marginal values in comparison to controls. The second set of quantifications, pooled together with the first set, did not result in a significant difference in bacterial load [Figure 5.2 B]. No haemocyte-specific knockdown had significantly different amounts of *M. marinum* in comparison to controls [Figure 5.2].

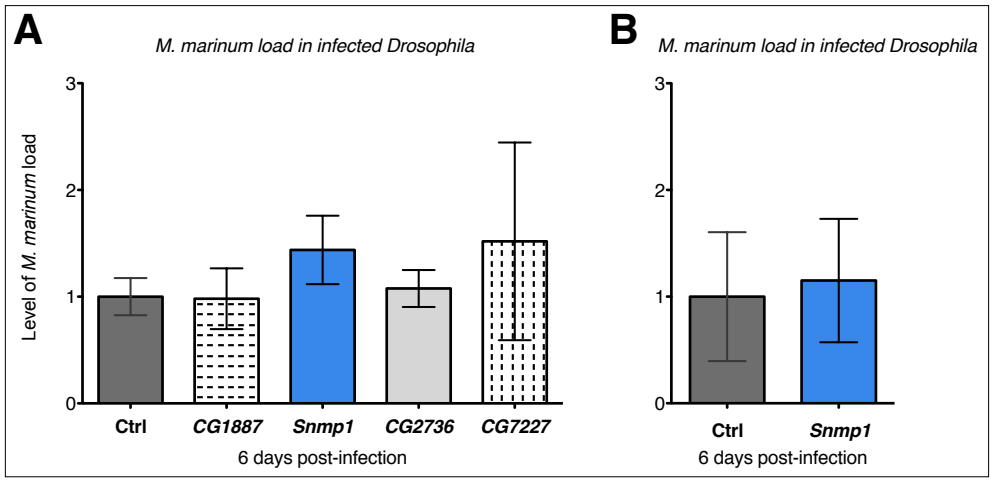


Figure 5.2 **Quantification of *M. marinum* load in infected adult *Drosophila* CD36 knockdown 6 days post-infection.** mRNA levels of *M. marinum*-specific gene *R8-9* were determined by qRT-PCR.
A.) CD36 gene knockdown and control (ctrl) *Drosophila* lines, the result is 1 experiment, n = 3 samples (9 males in total).
B.) *Snmp1* knockdown (2 ind. exp. including data from A), n = 7 (21 males in total).
Statistical significance between levels of *M. marinum R8-9* gene expression was determined using Mann-Whitney test (GraphPad Prism). Error bars represent standard deviation (SD).

5.2.2.3. *In vivo* imaging of uninfected *D. melanogaster*

A preliminary *in vivo* imaging screen included most of the *Drosophila* CD36 scavenger receptor genes and was performed using fluorescent microscopy. The preliminary microscopy screen did not reveal any obvious differences in phagocytosis of bacteria, nor in the progress of infection; however, this preliminary screen was done with speed in mind, and consistent imaging settings were being tested and established at that point. The obtained images are not of a high quality and are not shown apart from an example [Figure 5.3].

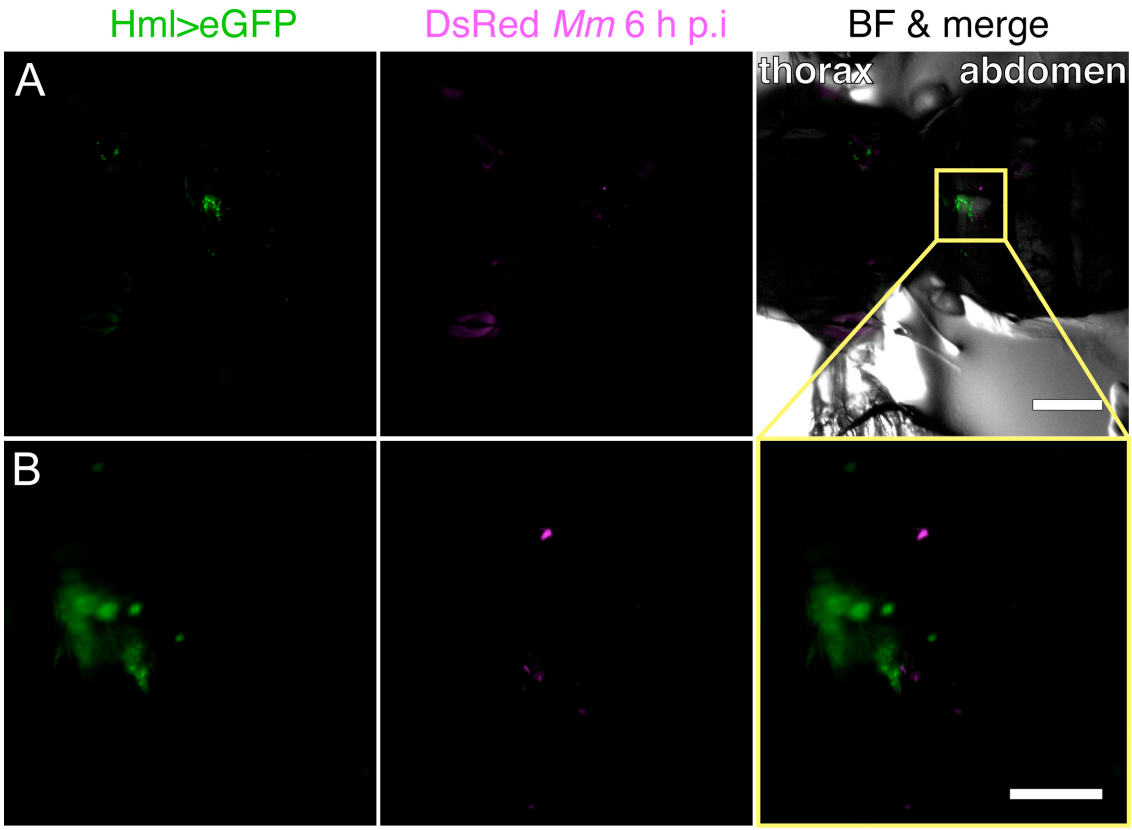


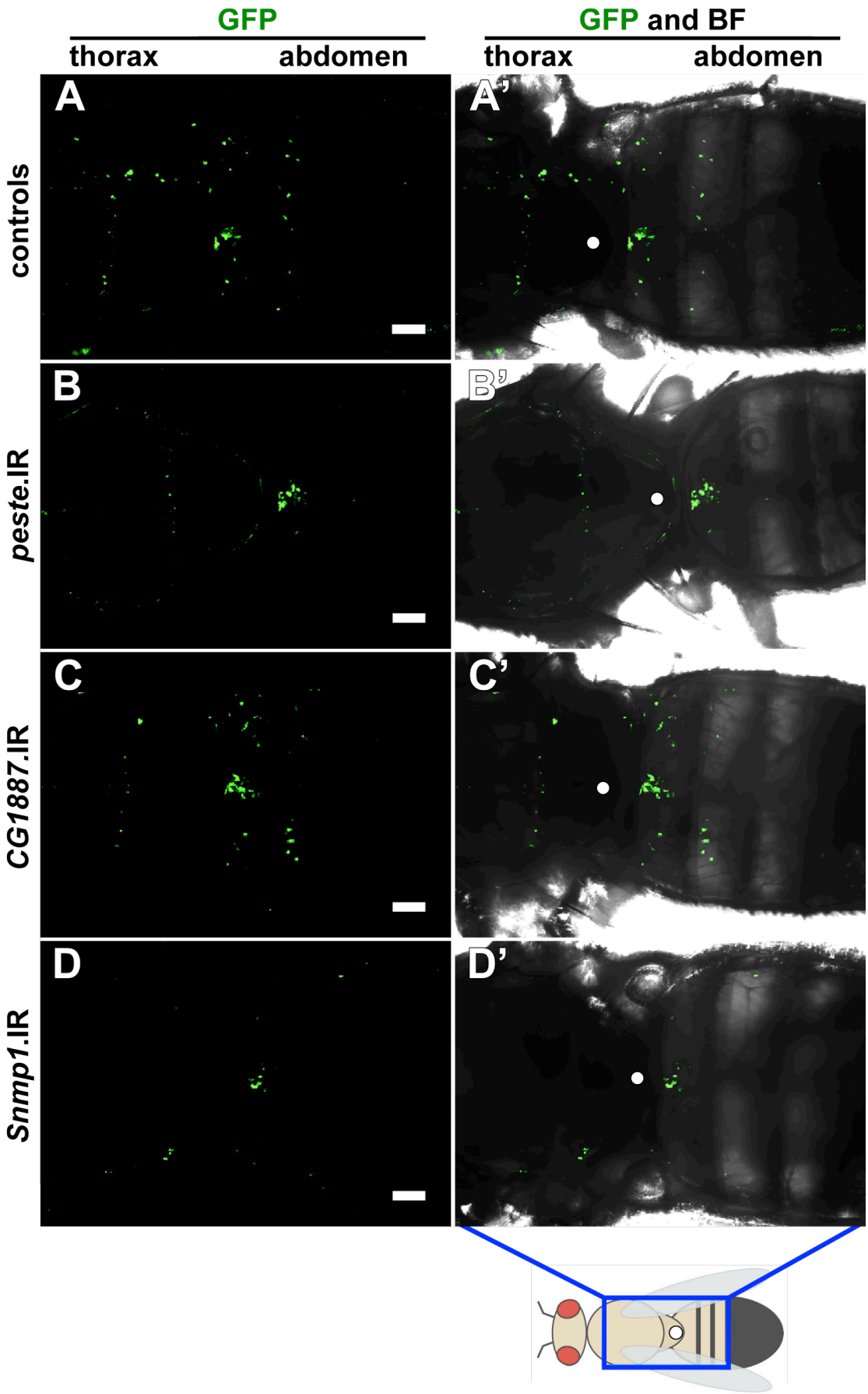
Figure 5.3 **Example of images obtained from preliminary screen of phagocytic receptors.** These images show a fly expressing eGFP in haemocytes that was also infected with DsRed *M. marinum* (*Mm*, magenta). The images show different magnification of the same fly. The scale bars represent 250 μm (A) and 75 μm (B).

Since no difference in the phagocytosis and progress of *M. marinum* infection was noted, untreated knockdown *Drosophila* was used for a more focused *in vivo* imaging screen using confocal microscopy to obtain a 'base line' of the distribution of adult haemocytes; only a subset of the CD36 RNAi lines was included. Haemocytes were imaged *in vivo* using eGFP expression under the control of *Hemolectin*, and in a limited location (dorsal abdomen and/or thorax) of untreated haemocyte-specific knockdown males. The *Snmp1* gene knockdown adult flies appeared to have fewer haemocytes, a phenotype that repeated in at least two independent experiments [Figure 5.4].

Haemocyte numbers of untreated males were also quantified using the automated counting feature of the Imaris software module. All samples were treated the same and a minimum of 3 imaged flies per genotype was used for the quantification. It appears that a haemocyte-specific knockdown of *Snmp1* results in fewer haemocytes, but the difference of quantified haemocytes was not significant [Figure 5.5].

RNAi lines targeting the other *Drosophila* CD36 scavenger receptors were not available at the time of this work: *croquemort* (*crq*, CG4280), *epithelial membrane protein* (*emp, CG2727*), *scavenger receptor acting in neural tissue and majority of rhodopsin is absent* (*santa-maria, CG12789*), *neither inactivation nor afterpotential D* (*ninaD, CG31783*), and *CG31741*.

Figure 5.4 ***In vivo*** **imaging of selected knockdown lines in uninfected state.** The location imaged was an area of the dorsal thorax and abdomen of untreated males.
A.) Driver-only control imaged using a green fluorescence channel (GFP); **A'.)** Driver-only control using bright field (BF) and GFP channel overlay.
B.) *peste* knockdown (GFP); **B'.)** *peste* knockdown (BF and GFP).
C.) *CG1887* knockdown (GFP); **C'.)** *CG1887* knockdown (BF and GFP).
D.) *Snmp1 knockdown* (GFP); **D'.)** *Snmp1 knockdown* (BF and GFP).
The imaged flies were alive and immobilised using cyanoacrylate glue at the time of imaging. Haemocytes are labelled with eGFP (green). The expression of eGFP was haemocyte-specific, using *Hml⊿* as a driver. Scale bars represent 100 μm. To ease identification of the border between the *Drosophila* thorax and abdomen (abd.), a white dot marks the tip of the fly notum in [A'], [B'], [C'] and [D']. The dot is also shown in the fly cartoon. The blue rectangle marks the imaged area.


GFP
GFP and BF
thorax
abdomen
thorax
abdomen
A
A'
controls
B
B'
peste.IR
C
C'
CG1887.IR
D
D'
Snmp1.IR

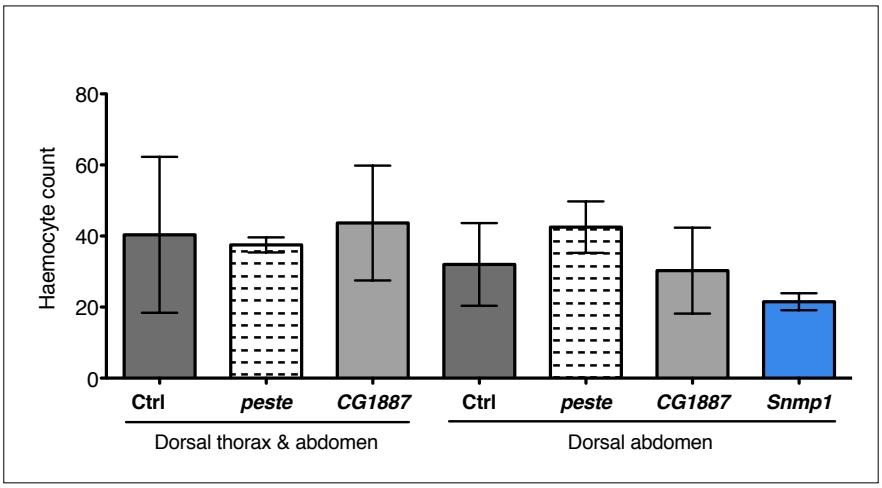


Figure 5.5 **Haemocyte numbers in untreated knockdown lines.** The data is based on two independent experiments; one shows haemocyte numbers in the dorsal thorax and abdomen (n = 3), and the second shows numbers in the dorsal abdomen only (n = 4). The graph represents the mean and SD. GraphPad Prism was used in statistical analysis, but no knockdown is significantly different from the controls.

5.2.3. *Drosophila* class C scavenger receptor family (SR-C)

The class CI *Drosophila* scavenger receptor (SR-CI) is involved in the phagocytosis of Gram-positive and Gram-negative bacteria *in vitro* (Pearson and Lux, 1995; Rämet *et al*., 2001). Although the other members are not known to have a direct link to infection and immunity in the fly, there is some evidence that even the *Sr-CII*, *Sr-CIII*, and *Sr-CIV* genes might be involved in immunity (Lazzaro *et al*., 2006). The scavenger receptors included in this screen were SR-CI, SR-CII, and SR-CIV.

***D. melanogaster scavenger receptor class C* genes**		
Gene name	**Gene abbreviation**	**Gene ID**
Scavenger receptor class C, type II	*Sr-CII*	*CG8856*
Scavenger receptor class C, type III	*Sr-CIII*	*CG31962*
Scavenger receptor class C, type IV	*Sr-CIV*	*CG3212*

Table 5.3 ***D. melanogaster* genes coding for the SR-C scavenger receptor family.** SR-C RNAi lines were used in this screen to reveal a *M. marinum*-specific phagocytic receptor in the fly.

5.2.3.1. Survival

RNAi lines targeting *Sr-CII* , *Sr-CIV* , and *Sr-CIII* were used to determine the potential role of these genes in survival after *M. marinum* infection. The haemocyte-specific knockdown of the *Sr-CIV* gene resulted in a significant increase in survival after *M. marinum* infection in comparison to controls [Figure 5.6 C]. The survival of the *Sr-CIII* and *Sr-CIII* gene knockdowns was not significantly different from driver-only controls [Figure 5.6 A and B]. The SR-C receptors were not included in the *in vivo* imaging part of this screen. The RNAi line targeting the *Sr-CI* (*CG4099*) gene was not available at the start of this screen.

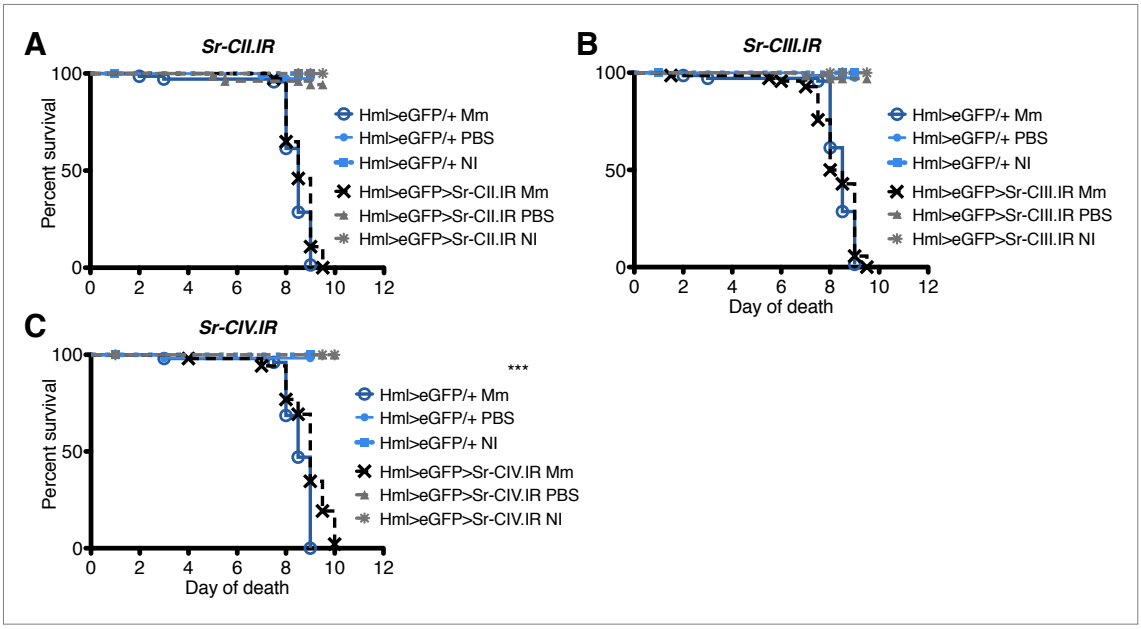


Figure 5.6 **Survival of *D. melanogaster* with haemocyte-specific knockdown of genes belonging to *Drosophila* class C scavenger receptors after *M. marinum* infection.** The fruit flies were infected with 500 CFU of WT *Mm*.
A.) *Sr-CII* knockdown (4 ind. exp.), n = min. 70 males.
B.) *Sr-CIII* knockdown (4 ind. exp.), n = min. 66.
C.) *Sr-CIV* (3 ind. exp.), n = min. 48.
Statistical significance between survival curves was determined using Log-rank analysis (Mantel-Cox, GraphPad Prism); *** $p < 0.0001$. Abbreviations: *Mm* – *M. marinum*, PBS – PBS-injected controls, NI – untreated controls, ind. exp. - independent experiments.

5.2.4. Phagocytic receptors

5.2.4.1. Survival

All *Drosophila* RNAi lines targeting the known and available *Drosophila* phagocytic receptors were included in a survival screen of *M. marinum*-infected *D. melanogaster*. The obtained data suggests that a haemocyte-specific knockdown of *nimA*, *nimC1*, *nimC2*, *nimC3*, *nimC4*, *eater*, *Dscam1*, *Dscam-like*, *Dscam3* makes no difference to *D. melanogaster* survival after *M. marinum* infection in comparison to controls. Only a subset of the obtained survival results is shown [Figure 5.7].

Miscellaneous *D. melanogaster* phagocytic receptor genes		
Gene name	**Gene abbreviation**	**Gene ID**
nimrod A	*nimA*	*CG42282*
nimrod A (2)	*nimA(2)*	*CG42282*
nimrod C1	*nimC1*	*CG8942*
nimrod C2	*nimC2*	*CG18146*
nimrod C3	*nimC3*	*CG16880*
nimrod C4	*nimC4*	*CG16876*
Eater	-	*CG6124*
Down syndrome cell adhesion molecule 1	*Dscam1*	*CG17800*
Down syndrome cell adhesion molecule-like	*Dscam-like*	*CG32387*
Down syndrome cell adhesion molecule 3	*Dscam3*	*CG31190*

Table 5.4 ***D. melanogaster* genes coding for phagocytic receptors.**

Apart from the survival assays, other experiments were done in parallel and revealed two phenotypes of *M. marinum*-infected flies that had specifically inactivated the phagocytic Nimrod C3 in the plasmatocytes. First, these flies had significantly lower bacterial load, and virtually no expression of the *Drosophila* antimicrobial peptide

Metchnikowin, and so the *nimC3* gene potential function in *M. marinum* infection was investigated further.

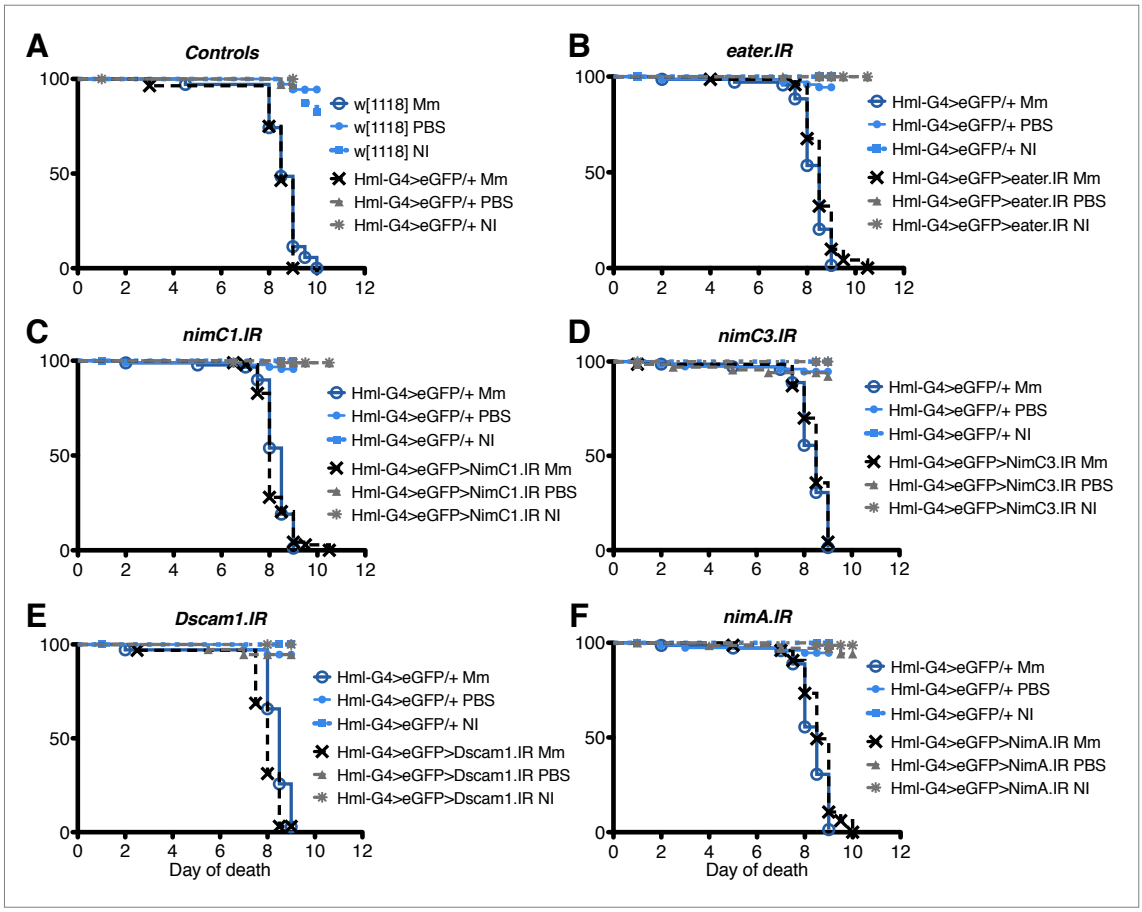


Figure 5.7 **Survival of *Drosophila* with haemocyte-specific knockdown of genes coding phagocytic receptors after *M. marinum* infection.** Only a subset of tested knockdown lines is shown. The fruit flies were infected with 500 CFU of WT *M. marinum*. Haemocyte-specific knockdown of the other tested genes gave similar results as shown in this figure.
A.) The survival of driver-only controls, *w[1118]; HmlΔGAL4,UAS-2xeGFP/+*, is not significantly different from that of *w[1118]* (DrosDel isogenic background). The result is based on 2 ind. exp., n = min. 28 males per genotype, per condition.
B.) *eater* knockdown (4 ind. exp.), n = min. 61.
C.) *nimC1* knockdown (5 ind. exp.), n = min. 87.
D.) *nimC3* knockdown (4 ind. exp.), n = min. 68.
E.) *Dscam1* knockdown (2 ind. exp.), n = min. 32.
F.) *nimA* knockdown (4 ind. exp.), n = min. 71.
Statistical significance between survival curves was determined using Log-rank analysis (Mantel-Cox, GraphPad Prism). The difference between survival curves was not significant. *Mm* – *M. marinum*, PBS – PBS-injected controls, NI – untreated controls, ind. exp. - independent experiments.

5.2.5. The *Drosophila* Nimrod C3

The *Drosophila* Nimrod C3 phagocytic receptor was studied to determine if it played a role in *M. marinum* infection of the fly. The gene *nimC3* was knocked down in a macrophage-specific manner; plasmatocyte is the *Drosophila* macrophage (Rämet *et al*., 2001). The function of *nimC3* in *M. marinum* infection was examined by survival assays, bacterial load measurement, and by testing the expression levels of antimicrobial peptides (AMPs) and haemocyte-specific markers (*Hemolectin*, *croquemort*).

When the *nimC3* gene was silenced in plasmatocytes, survival of *M. marinum*-infected animals was not affected. The survival assay was repeated 4 times and the total sample size was at least 68 male flies per genotype, per condition [Figure 5.7 D]. Control flies were mock-infected (PBS) or untreated (NI), and were alive at least for the whole duration of this experiment.

5.2.5.1. Bacterial load

Despite not having any survival phenotype, the *nimC3* knockdowns had fewer bacteria at 6 days post-infection. Together with other phagocytic gene knockdown lines, *nimC3* knockdown was used to examine the level of *M. marinum* load at 6 days post-infection. The RNAi targeting *nimC1*, *nimC2*, *nimC3*, *eater* and *Dscam3* were tested, but only *nimC3* line was tested twice. Details about other genes mentioned and shown in [Figure 5.8] can be found in chapter 5. Haemocyte-specific knockdown of *nimC3* led to a significantly reduced bacterial load of *M. marinum* at 6 days after infection [Figure 5.8 B]. The level of bacterial load in *nimC3* knockdowns was quantified from two independent experiments, giving the sample size of 7, which equals to 21 animals analysed in total. In comparison to controls, haemocyte-specific knockdown of *nimC3* resulted in significantly ($p < 0.001$) lower load of *M. marinum* at 6 days post-infection.

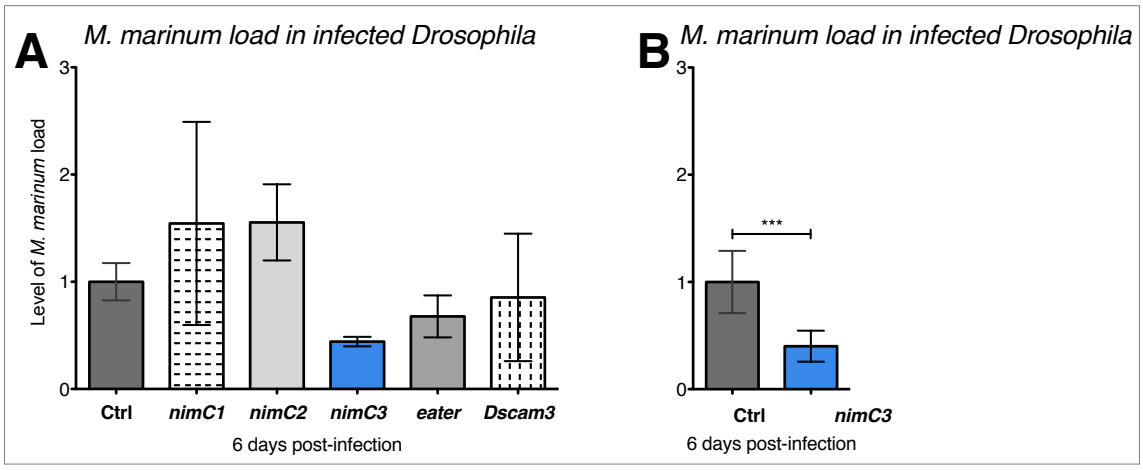


Figure 5.8 **Quantification of *M. marinum* load in infected adult *Drosophila* with *nimC3* knockdown at 6 days post-infection.** mRNA levels of *M. marinum*-specific gene *R8-9* were determined by qPCR.
A.) mRNA was quantified in several knockdown lines, and in driver-only controls. Data is based on 1 experiment, n = 3 samples (= 9 animals per genotype, per condition).
B.) *nimrod C3* knockdown, data is based on 2 independent experiments (incl. data from [A]), n = 7 samples (= 21 animals).
The error bars represent standard deviation (SD). Statistical significance between the levels of *M. marinum R8-9* expression was determined using Mann-Whitney test (GraphPad Prism), *** $p < 0.001$.

5.2.5.2. Quantification of haemocytes in *M. marinum*-infected *D. melanogaster*

In a preliminary *in vivo* imaging screen it appeared that *nimC3* knockdown had fewer haemocytes (not shown). qRT-PCR was used as a quantifying method for haemocyte-specific markers – *Hemolectin* (*Hml*) and *croquemort* (*crq*) – to indicate the numbers of haemocytes in infected and control flies (Defaye *et al*., 2009; Franc *et al*., 1996). However, since I obtained these results, other studies have shown that *crq* expression may not be haemocyte specific in the adult fly (Clark *et al*., 2011; McQuilton *et al*., 2012).

The *nimC3* knockdown was driven with the *Hemolectin* promoter (*HmlΔ*). Hml is depleted in *M. marinum*-infected *nimC3* knockdown and control flies, whereas crq levels are more or less constant [Figure 5.9]. It is not possible to conclude whether the Hml reduction is specific to *M. marinum* infection, because a control bacterium was not used; however, it had been shown that 1. the then known haemocyte-specific markers, such as *HmlΔ*, *Dpp* and *crq*, do not overlap and co-express 100% (Clark *et al*., 2011); 2. crq is not haemocyte-specific. Therefore, it is highly likely that *crq-GAL4* expression in [Figure 5.9] is unchanged because *crq* may be expressed by Hml^{-ve} haemocytes or crq^{+ve} cells or both. There is a possibility that *nimC3* levels are low because *M. marinum* infection inhibits *Hml* expression and since *HmlΔ* is the driver of this particular knockdown, the level of *Hml* in *nimC3* is falsely low. However, it is also possible that Hml^{+ve} haemocytes are killed or severely affected by the infection. To confirm this data, it is necessary to use other haemocyte-specific driver to knockdown *nimC3* expression.

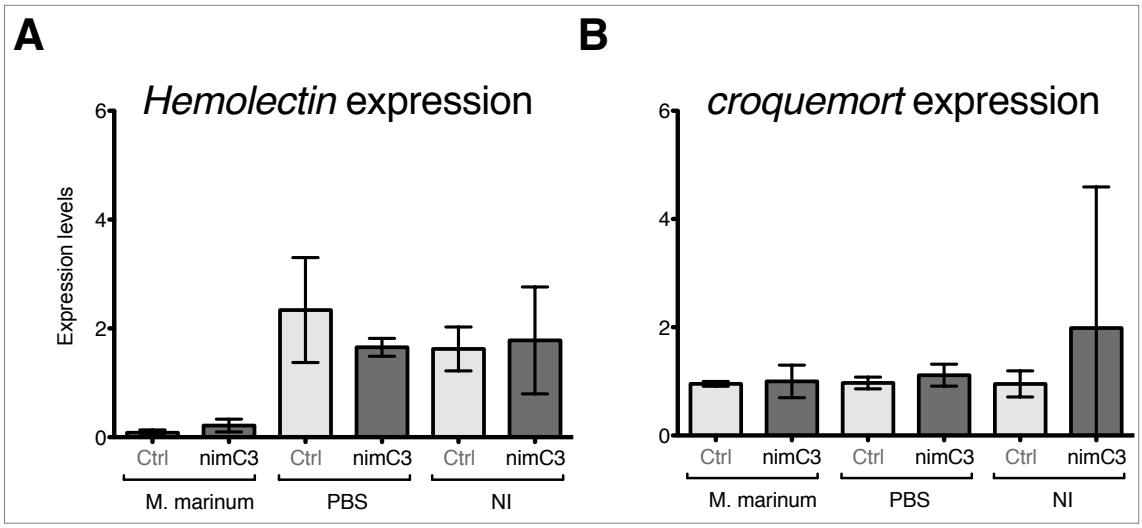


Figure 5.9 ***D. melanogaster*** **haemocyte-specific marker expression in infected and control animals at 6 days post-infection.**

A.) Expression levels of *Hml* in *M. marinum*-infected and control *nimC3* knockdown flies are not significantly different from those of the control flies. However, the expression of *Hml* is significantly reduced in infected flies. The knockdown was driven using *HmlΔGAL4*. *M. marinum* and PBS data is based on 2 independent experiments; 7 samples were used (21 animals). NI controls are based on 1 experiment only, n = 4 samples (= 12 animals).

B.) Expression of the *crq* gene in *M. marinum*-infected and control *nimC3* is not significantly different from that of the controls. The knockdown was driven using *HmlΔGAL4*. The data is based on 1 experiment, n = 4 samples (= 12 animals per genotype, per condition). Statistical significance between samples was determined using Mann-Whitney test (GraphPad Prism); error bars represent SD.

5.2.5.3. AMP induction in haemocyte-specific *nimC3* knockdown after *M. marinum* infection

Infection induces the expression of *Drosophila* antimicrobial peptides (Lemaitre and Hoffmann, 2007). The main known *Drosophila* AMPs were quantified using qRT-PCR in the infected and control flies [Figure 5.10], but levels of only one of them – the antifungal Metchnikowin – were significantly different from controls [Figure 5.10 B]. The AMP levels were quantified on one occasion, the total number of animals tested per sample was 3, and 7 samples were analysed (21 animals in total). The variability observed in the control is typical of what we see with *M. marinum* induction of antimicrobial peptides.

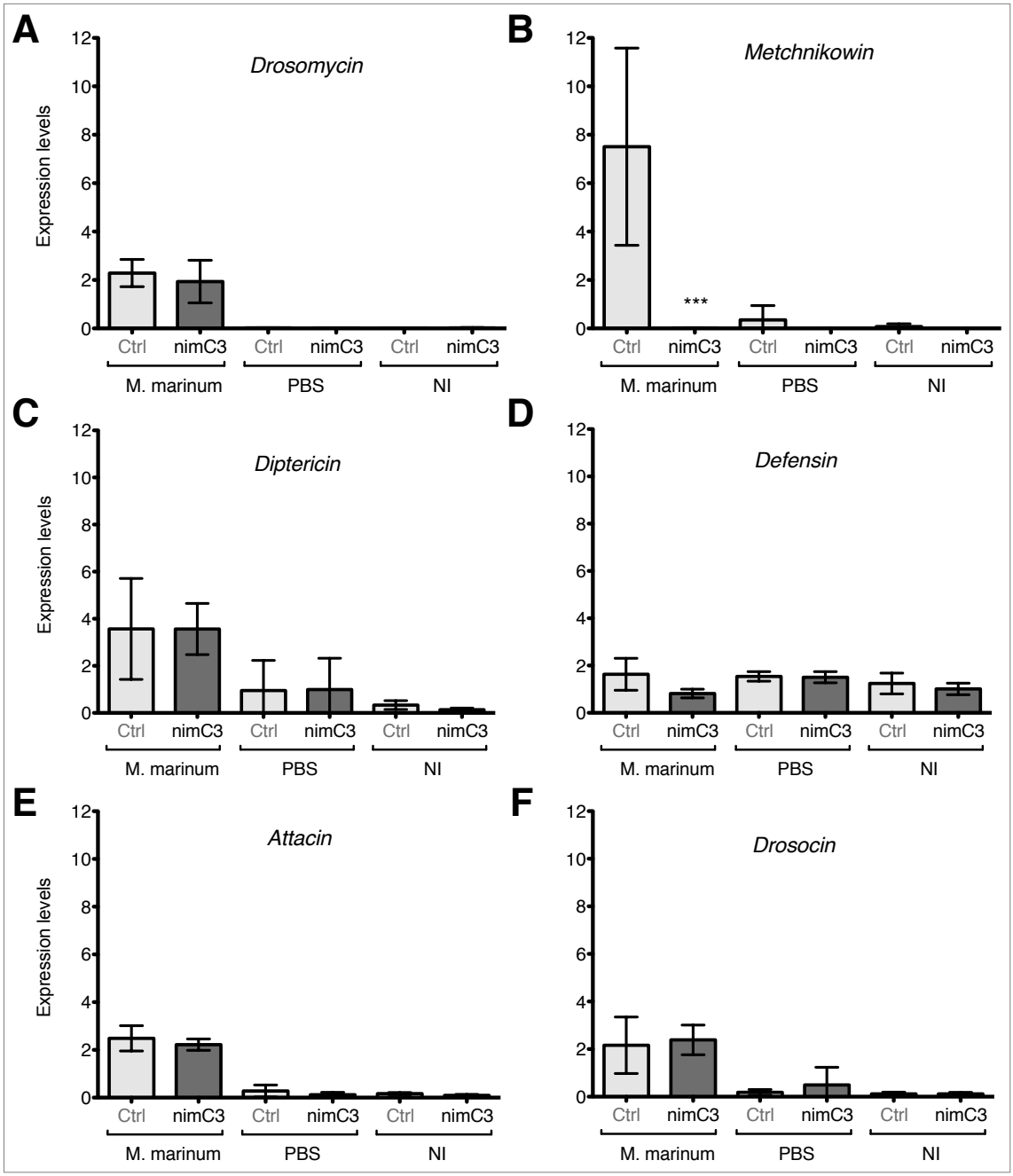


Figure 5.10 **The expression of AMP Metchnikowin is significantly reduced in *M. marinum*-infected *nimC3* knockdown *D. melanogaster* at 6 days post-infection.** Controls were either mock infected (PBS) or untreated (NI). Levels of AMP mRNA were determined by qRT-PCR.
A.) *Drosomycin;* **B.)** *Metchnikowin;* **C.)** *Diptericin;* **D.)** *Defensin;* **E.)** *Attacin;* **F.)** *Drosocin*.
Statistical significance between levels of *Metchnikowin* expression was determined using Mann-Whitney test (GraphPad Prism); *** $p < 0.001$. The data is based on 1 experiment, n = 4 samples (= 12 animals per genotype, per condition), except in the case of Metchnikowin, which was analysed on 2 separate occasions; n = 7 (= 21 animals). Error bars represent SD.

5.2.5.4. Age-specific *Hemolectin* and *croquemort* expression in untreated *nimC3* knockdown *D. melanogaster*

Since the expression of *Hml* and *crq*, as haemocyte markers, was different in *M. marinum* flies [Figure 5.9], I quantified levels of these markers by qRT-PCR in untreated animals of different ages, 1 – 25 days old. Although this experiment was done once, it appears that the expression of these markers differs little in adult males [Figure 5.11 A and B]. The driver used to drive the RNAi targeting *nimC3* was *HmlΔ*. *Hemolectin* levels were quantified in animals 5 – 15 days old twice [Figure 5.11 C]. From the data it appears that the expression of *Hemolectin* is slightly decreased in 10-day old *nimC3* knockdown *Drosophila*.

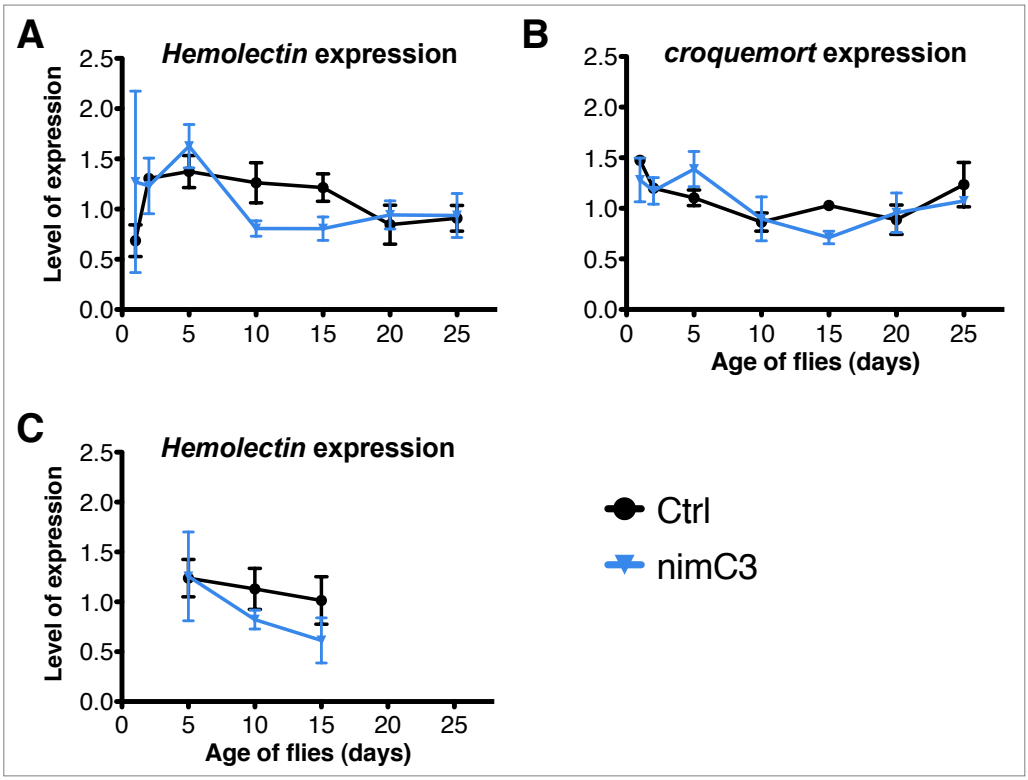


Figure 5.11 ***Hml* and *crq* levels in untreated animals of different ages.**
A.) *Hemolectin* levels
B.) *croquemort* expression. The data in [A] and [B] is based on 1 experiment, n = 3 samples (= 9 animals per genotype, per condition).
C.) *Hml* expression data is based on 2 experiments (including data from A), n = 7 samples (= 21 animals per genotype, per condition).
The RNAi was driven using *HmlΔGAL4* driver. Error bars represent SD.

5.2.5.5. *In vivo* imaging of uninfected *Drosophila*

Following a preliminary screen that did not show any difference in phagocytosis nor bacterial spread, I imaged only untreated animals to see the typical distribution of adult haemocytes, and possibly find a link between the low load of *M. marinum* in the *nimC3* gene knockdown flies. Haemocyte numbers were not obviously different between the gene knockdowns and control flies. Only controls and the *nimC3* knockdown are shown [Figure 5.12 and Figure 5.13].

Haemocyte numbers of untreated males were also quantified using the automated counting feature of the Imaris image processing and analysis software. All images were processed using identical settings, and a minimum of 3 imaged flies per genotype was used for the quantification. The difference in quantified haemocytes between controls and *nimC3* knockdown lines was not significant [Figure 5.14].

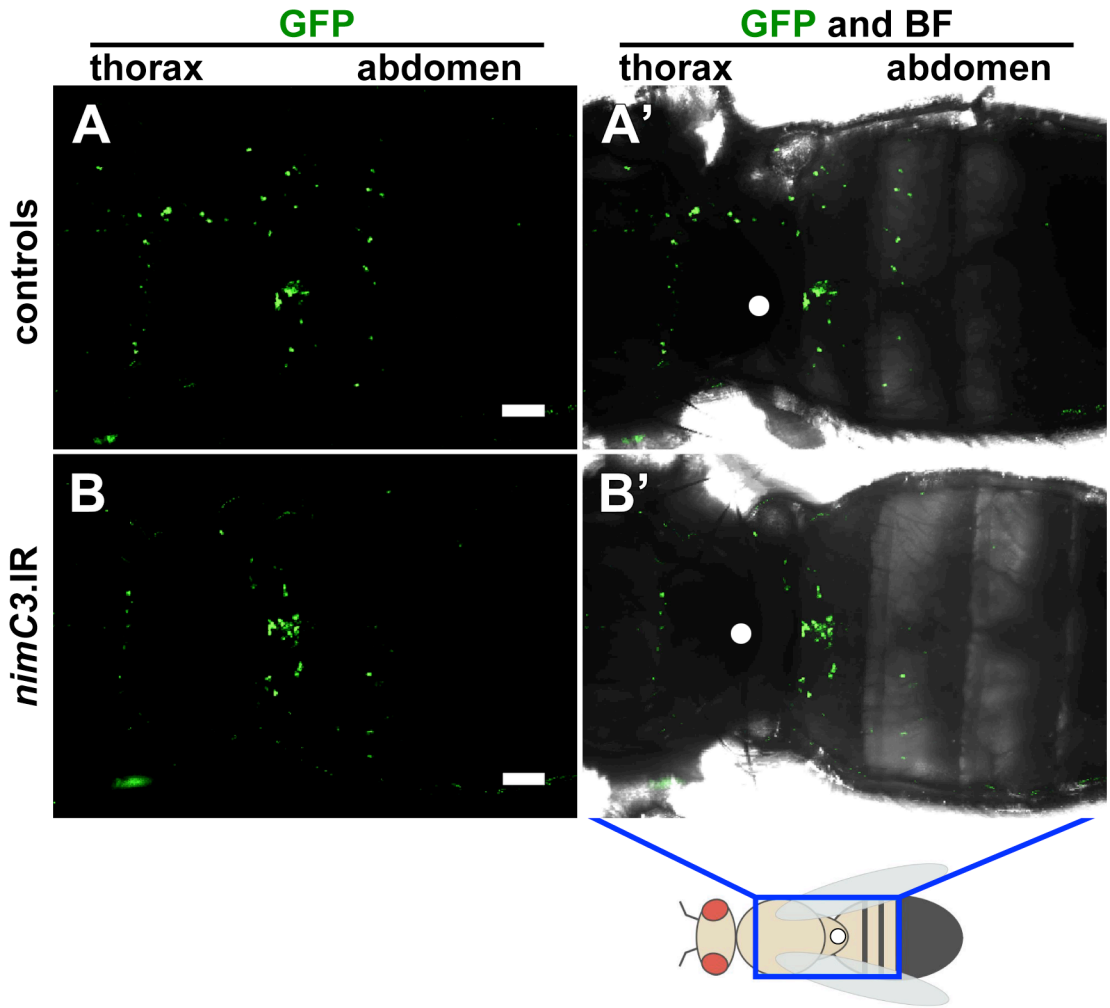


Figure 5.12 ***In vivo* imaging of *nimC3* knockdown line in uninfected state.** The location imaged was an area of the dorsal thorax and abdomen of untreated males.
A.) Driver-only controls imaged using a green fluorescence channel (GFP).
A'.) Driver-only control using bright field (BF) and GFP channel overlay.
B.) *nimC3* knockdown (GFP).
B'.) *nimC3* knockdown (BF and GFP).
The imaged flies were alive and immobilised using cyanoacrylate glue at the time of imaging. Haemocytes are labelled with eGFP (green). The expression of eGFP was haemocyte-specific, using *HmlΔ* as a driver. Scale bars represent 100 μm. The white dot marks the tip of the fly notum in [A'] and [B']. The dot is also shown in the fly cartoon. The blue rectangle marks the imaged area.

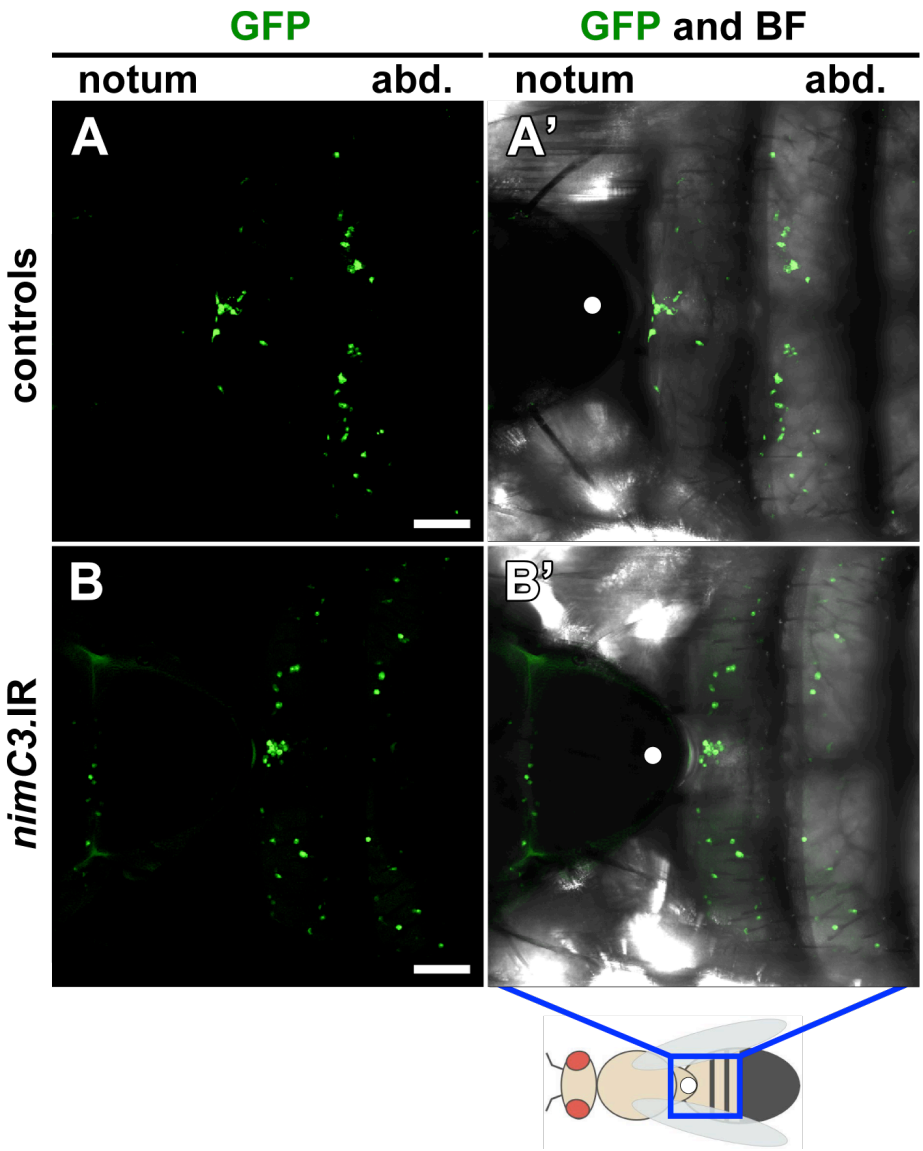


Figure 5.13 ***In vivo*** **imaging of dorsal abdomen of untreated *nimC3* knockdown.**
A.) Driver-only control imaged using a green fluorescence channel (GFP)
A'.) Driver-only control using bright field (BF) and GFP channel overlay.
B.) *nimC3* knockdown (GFP)
B'.) *nimC3* knockdown (BF and GFP). Haemocytes were labelled with eGFP (green).
The expression of eGFP was haemocyte-specific, using *HmlΔ* as a driver. Scale bars represent 100 μm. A white dot marks the tip of the fly notum in [A'] and [B']. The dot is also shown in the fly cartoon. The blue rectangle marks the imaged area.

The figures [Figure 5.12] and [Figure 5.13] are very similar; however, they were obtained on two different occasions, and both sets were used for the counting of haemocytes. Both figures are included in order to show exactly what regions of the fly were used to count haemocytes. Hence the "dorsal thorax & abdomen" and "dorsal abdomen" are distinguished in [Figure 5.14].

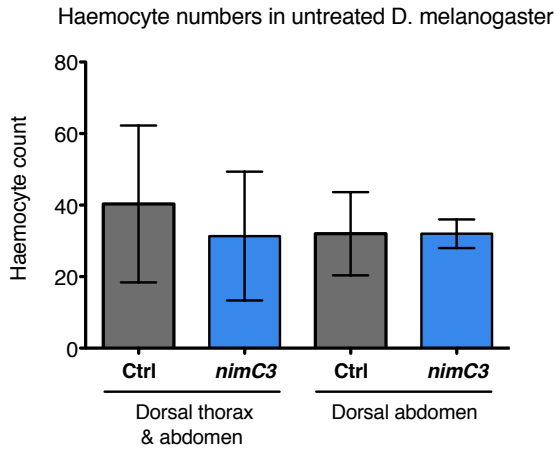


Figure 5.14 **Haemocyte count is not significantly different in untreated *nimC3* knockdown flies in comparison to controls.** The data is based on two independent experiments. The left two columns show haemocyte numbers in the dorsal thorax and abdomen (n = 3 animals). Haemocytes were counted from the samples shown in [Figure 5.12]. The right two columns show numbers in the dorsal abdomen only (n = 4). Haemocytes were counted from the samples shown in [Figure 5.13]. Error bars represent the SD. The difference between controls and *nimC3* knockdown animals was not significant (GraphPad Prism).

5.2.5.6. Haemocytes of animals with the inactivated *nimC3* gene phagocytosed pHrodo-labelled *E. coli*

pHrodo-labelled *E. coli* (pHrodo) is a rhodamine sensor of pH that becomes fluorescent in an acidic environment. Thus, pHrodo becomes fluorescent if it is phagocytosed into an acidified compartment. *In vivo* imaging shows that *nimC3* knockdown appear to have normal phagocytic function [Figure 5.15 D]. The image shows part of the *Drosophila* dorsal abdomen and a small portion of the thorax (notum). pHrodo-injected animals

[Figure 5.15 C and D] are compared to uninjected flies [Figure 5.15 A and B], and also to each other. The control flies (ctrl) carry only the driver *HmlΔGAL. Drosophila* plasmatocytes are labelled in green; pHrodo in magenta.

Although no study had been previously done to test the specificity of the Nimrod receptors for dead *E. coli*, NimC1 had been shown to be specific for *S. aureus* in S2 cells, and to have a possible redundant role in the uptake of *E. coli*. (Kurucz *et al*., 2007). Results described in this chapter suggest that NimC3 is not required for the uptake of dead *E. coli* (as a part of pHrodo) [Figure 5.15].

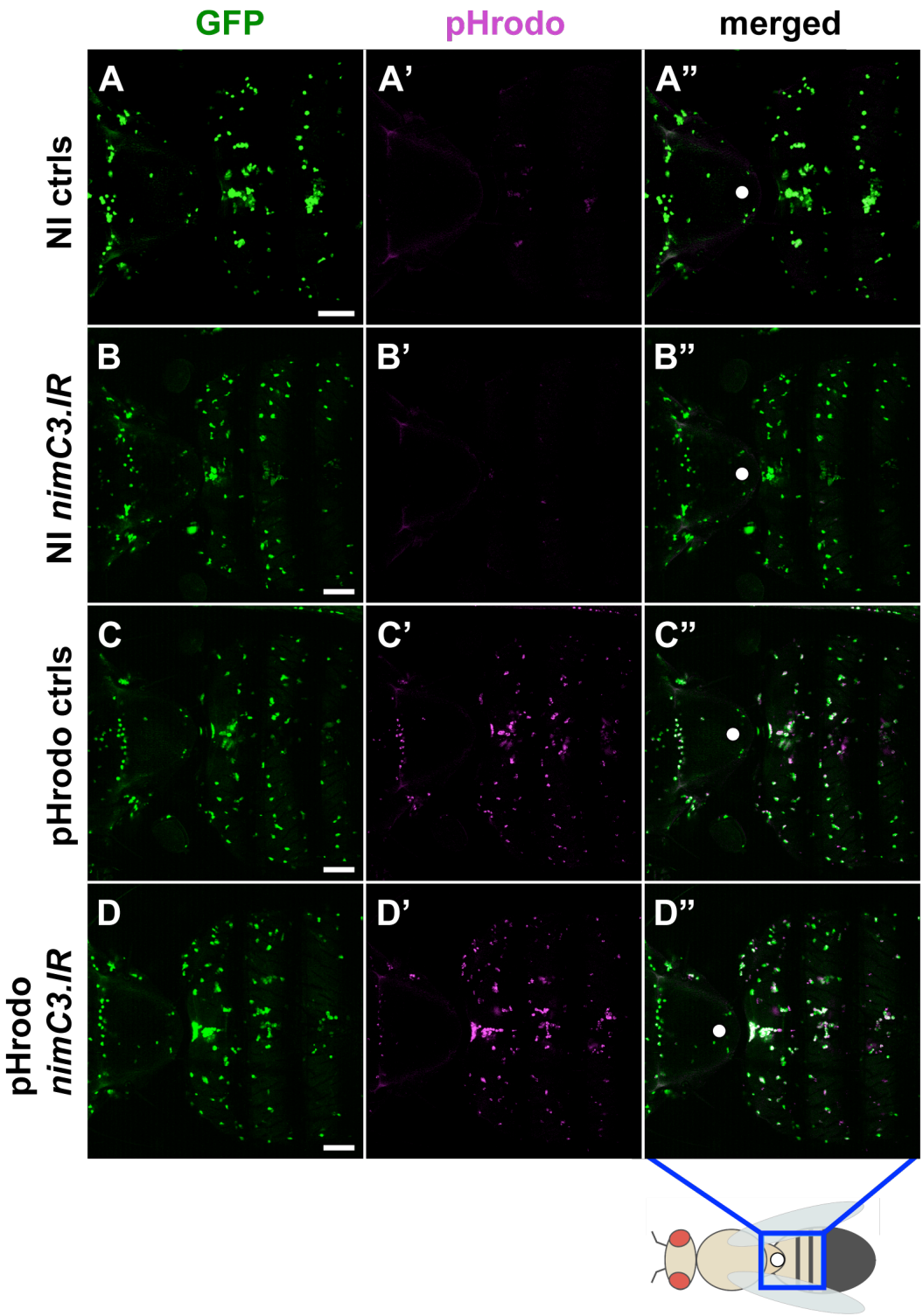


Figure 5.15 **Haemocyte phagocytic function appears normal in *nimC3* knockdown.**
A.) Untreated driver-only controls. Image is representative of at least 3 samples.
B.) Untreated *nimC3* knockdown. At least 3 animals were imaged.
C.) pHrodo-injected driver-only controls. At least 3 animals were imaged.
D.) pHrodo-injected *nimC3* knockdown. At least 3 animals were imaged.
The white dots mark the fly notum. RNAi was haemocyte-specific using *HmlΔ*.
Scale bars represent 100 μm.

5.3. Conclusion

The function of various phagocytic and scavenger receptors in connection with *M. marinum* infection in the fly was tested by using several methods – survival assays, bacterial load, and *in vivo* imaging of selected RNAi lines. All the tested knockdowns were haemocyte-specific, using *Hml∆* as a driver.

Survival assays showed that the haemocyte-specific knockdown of some genes had an effect on survival in *M. marinum*-infected flies. The following RNAi lines were significantly longer-lived: *Snmp1*, *CG3829*, *CG1887*, *CG2736*, *CG10345*, *CG7227*, and *Sr-CIV*. However, it is not clear whether the knockdown of these genes results in a *M. marinum*-specific phenotype and this needs to be investigated further.

The bacterial load of *M. marinum* was tested only in selected RNAi lines, and out of these, only one line - *nimC3* – resulted in a significantly lower *M. marinum* load at 6 days post-infection. The gene for phagocytic receptor *nimC3* does not appear to play a role in survival following *M. marinum* infection. However, the *nimC3* knockdown animals had a couple of phenotypes when the RNAi was driven with *Hml∆*. The expression of antifungal microbial peptide Metchnikowin is virtually blocked in the *nimC3* knockdown flies, infected or uninfected, and *M. marinum* numbers are significantly lower in the infected knockdown flies in comparison to controls [Figure 5.8]. The latter phenotype should be tested using other bacterium to see if the lower load is *M. marinum*-specific in these knockdowns. Following the *M. marinum* load experiment, pHrodo-labelled *E. coli*, a rhodamine sensor of pH, was injected into flies, knockdown and control to test the function of phagocytic cells. The phagocytes function and ingest pHrodo even if the *nimC3* gene is knocked down. Also, *Hemolectin* expression appeared lower at 10 days old animals in comparison to controls, but it is not

clear if there are any connection between the *Hemolectin* levels and low *M. marinum* numbers in infected flies.

Although the preliminary *in vivo* imaging screen revealed a 'fewer-plasmatocyte' phenotype in untreated *nimC3* knockdown adults, this phenotype did not repeat. When an expression of the plasmatocyte markers, *Hemolectin* and *croquemort*, was tested in infected males, there was an obvious reduction of Hml expression in infected flies at 6 post-infection, but no change of *crq* expression was noted. *In vivo* imaging did not show any difference between the *nimC3* knockdown and controls [Figure 5.12 and Figure 5.13].

In the future, another type of haemocyte-specific driver, and loss-of-function mutants of the tested genes, would be required to validate the data obtained in this study. Additionally, control bacteria, pathogenic and non-pathogenic, would have to be tested alongside *M. marinum* to determine if any of the tested phagocytic receptors were mycobacterium-specific *in vivo*.

Chapter 6. *BURKHOLDERIA THAILANDENSIS* IS VIRULENT IN *DROSOPHILA MELANOGASTER*

Abstract

Melioidosis is a serious infectious disease endemic to Southeast Asia and Northern Australia. This disease is caused by the Gram-negative bacterium *Burkholderia pseudomallei; Burkholderia thailandensis* is a closely related organism known to be avirulent in humans. *B. thailandensis* has not previously been used to infect *Drosophila melanogaster*. We examined the effect of *B. thailandensis* infection on fly survival, on antimicrobial peptide expression, and on phagocytic cells. In the fruit fly, which possesses only an innate immune system, *B. thailandensis* is highly virulent, causing rapid death when injected or fed. One intriguing aspect of this infection is its temperature dependence: infected flies maintained at 25 °C exhibit rapid bacterial proliferation and death in a few days, while infected animals maintained at 18 °C exhibit very slow bacterial proliferation and take weeks to die; this effect is due in part to differences in immune activity of the host. Death in this infection is likely due at least in part to a secreted toxin, as injection of flies with sterile *B thailandensis*-conditioned media is able to kill. *B. thailandensis* infection strongly induces the expression of antimicrobial peptides, but this is insufficient to inhibit bacterial proliferation in infected flies. Finally, the function of fly phagocytes is not affected by *B. thailandensis* infection. Together, our data indicate that the interaction between insects and *Burkholderia thailandensis* is complex; the high virulence of *B thailandensis* in the fly suggests the possibility that this organism is a natural pathogen of one or more invertebrates.

6.1. Introduction

Melioidosis is a serious human and animal disease caused by the Gram-negative bacterium *Burkholderia pseudomallei* (*B. pseudomallei*). Moist soils of rice paddies or surface water harbour this pathogen in endemic areas of Southeast Asia and Northern Australia (Chaowagul *et al*., 1989; Cheng and Currie, 2005; Smith *et al*., 1995). Melioidosis can be contracted through damaged skin from *B. pseudomallei*-infected soil and water or by inhaling aerosolised bacteria (White, 2003). In humans, melioidosis can manifest itself as a fever, mild or severe septicaemic pneumonia, skin and internal organ abscesses, and neurological conditions, such as brainstem encephalitis (Chaowagul *et al*., 1989; Currie *et al*., 2000b). The treatment of melioidosis is long and frequently unsuccessful; in many cases the disease recurs (Chaowagul *et al*., 1993). Currie and colleagues conducted a 10-year study of melioidosis patients and found that approximately 86% of patients who suffer septic shock as a result of this infection die (Currie *et al*., 2000a). The outcome of melioidosis also depends on individual circumstances and risk factors; diabetes, chronic renal disease or alcoholism have been reported to increase the rate of death in melioidosis patients (Currie *et al*., 2000a; Suputtamongkol *et al*., 1999).

B. pseudomallei infection has been studied in Syrian golden hamsters to model melioidosis; in mice to understand various aspects of the bacterial pathogenicity, such as the effect of wild-type (WT) or mutant strains of *B. pseudomallei* on the survival of WT mice, and *in vitro* to gain insight into the intracellular life cycle of *B. pseudomallei* and its motility (Brett *et al*., 1997; Pilatz *et al*., 2006; Stevens *et al*., 2005a). As this highly pathogenic bacterium is a Class B infectious agent, its study requires BSL-3 containment conditions (Rotz *et al*., 2002). In addition, *B. pseudomallei* is resistant to

many antibiotics; restrictions on the use of antibiotics in the study of this pathogen apply (Schweizer and Peacock, 2008; Vorachit *et al*., 1993). Due to these limitations, a safer and cheaper model for the study of some aspects of melioidosis could prove invaluable.

B. pseudomallei is closely related to the non-pathogenic *Burkholderia thailandensis* (*B. thailandensis*) (Brett *et al*., 1998; Kim *et al*., 2005; Yu *et al*., 2006). When discovered, *B. thailandensis* was thought to be an isolate of *B. pseudomallei*; later Brett and colleagues renamed it from *B. pseudomallei*-like to its current name (Brett *et al*., 1998). Although *B. thailandensis* is mostly avirulent in mammals, high doses of *B. thailandensis E264* kill mice (Haraga *et al*., 2008; Wiersinga *et al*., 2008). *B. thailandensis* and *B. pseudomallei* are motile, and live in soil and surface water, and are therefore adapted to similar environmental conditions (Kespichayawattana *et al*., 2000; Stevens *et al*., 2005a; Stevens *et al*., 2005b). Although *B. thailandensis* is not virulent in the Syrian golden hamster model (Brett *et al*., 1997), occasional *B. thailandensis* infections have been reported in people; in 1999 a motorcycle accident in Thailand led to melioidosis-like symptoms (here *B. thailandensis* is referred to as Ara+ *B. pseudomallei)* (Lertpatanasuwan *et al*., 1999); in the U.S., Glass and colleagues reported that *B. thailandensis* strain ATCC 700388 infection led to pneumonia and septicaemia in a 2-year old boy involved in a car accident (Glass *et al*., 2006).

Drosophila melanogaster (*D. melanogaster*) is a proven model for the study of various infections, such as *Mycobacterium marinum* (Dionne *et al*., 2003), *Salmonella typhimurium* (Brandt *et al*., 2004), and *Burkholderia cepacia* (Castonguay-Vanier *et al*., 2010). Despite the fact that no adaptive immunity has been discovered in *D. melanogaster*, the fly is an attractive potential model host to examine the role of innate immunity in melioidosis.

Drosophila melanogaster (*D. melanogaster*) is a proven model for the study of various infections, such as *Mycobacterium marinum* (Dionne *et al*., 2003), *Salmonella typhimurium* (Brandt *et al*., 2004), and *Staphylococcus aureus* (Needham *et al*., 2004). The interactions of *Drosophila* with the *Burkholderia cepacia* complex have also been previously examined (Castonguay-Vanier *et al*., 2010; Schneider *et al*., 2007). However, to our knowledge, non-cepacia *Burkholderiaceae* have not previously been examined in *Drosophila*, despite the appeal of this organism as a potential model host to examine the role of innate immunity in melioidosis.

The aim of this study was to evaluate *D. melanogaster* as a model organism for the study of host-pathogen interactions and the role of the innate immune response in melioidosis. The results show that *B. thailandensis* infection in *D. melanogaster* to some extent parallels *B. pseudomallei* infection in mammalian hosts. This model thus may advance our understanding of the host-pathogen interaction in terms of innate immunity.

6.2. Results

6.2.1. *B. thailandensis E264* is pathogenic in *Drosophila melanogaster*

Burkholderia thailandensis E264 (*B. thailandensis*) is avirulent in people under normal conditions; however, it is highly pathogenic in wild-type (*Oregon R*) *D. melanogaster* (5-10 days old). 100% of flies injected with *B. thailandensis* died reliably within 3.5 days of infection [Figure 6.1 A] and increasing bacterial dose resulted in more rapid mortality [Figure 6.8 A]. The survival assays were repeated several times using only the lowest bacterial dose (OD_{600} of 0.01). We also tested w^{1118} males (DrosDel isogenic background) to see if the effect of *B. thailandensis* infection was the same as it had been in *Oregon R* flies. The survival data is consistent in both genotypes [Figure 6.8 B]. Finally, this lethality required live bacteria: heat-killed *B thailandensis* did not cause lethality [Figure 6.8 C].

To ensure that the mortality was caused by *B. thailandensis* and not by septic injury per se, we tested another Gram-negative bacterium, *E. coli* (*DH5α*), as a negative control for *B. thailandensis* infection in wild-type (*Oregon R*) flies. *E. coli* infection was not lethal in flies, which was consistent with previous studies (Pimenta *et al*., 2003; Schneider and Shahabuddin, 2000), whereas *B. thailandensis* killed consistently within 3.5 days of infection [Figure 6.1 B]. This experiment had another purpose - to test if infection with calibrated but pure *B. thailandensis* culture, in its original growth medium and unwashed with PBS, was as pathogenic as 'washed' culture (resuspended in PBS). The difference between the survival curves was not significant [Figure 6.8 D]; all infected flies were dead within 3.5 days of *B. thailandensis* infection.

We next wanted to test whether the observed lethality was accompanied by bacterial proliferation. We analysed *B. thailandensis* growth in infected flies by homogenising

them in PBS at 0, 6 and 24 hours p.i. and counting viable bacterial colonies. *B. thailandensis* survived in the fly; an initial phase of low growth between 0 and 6 hours after infection was followed by rapid bacterial proliferation [Figure 6.1 C].

B. thailandensis E264 is thus a highly virulent pathogen in *Drosophila*, with a low dose (~250 CFU per fly) leading to rapid death of the host. For subsequent experiments, we have focused on the effects of the lowest verified infectious dose (OD_{600} of 0.01).

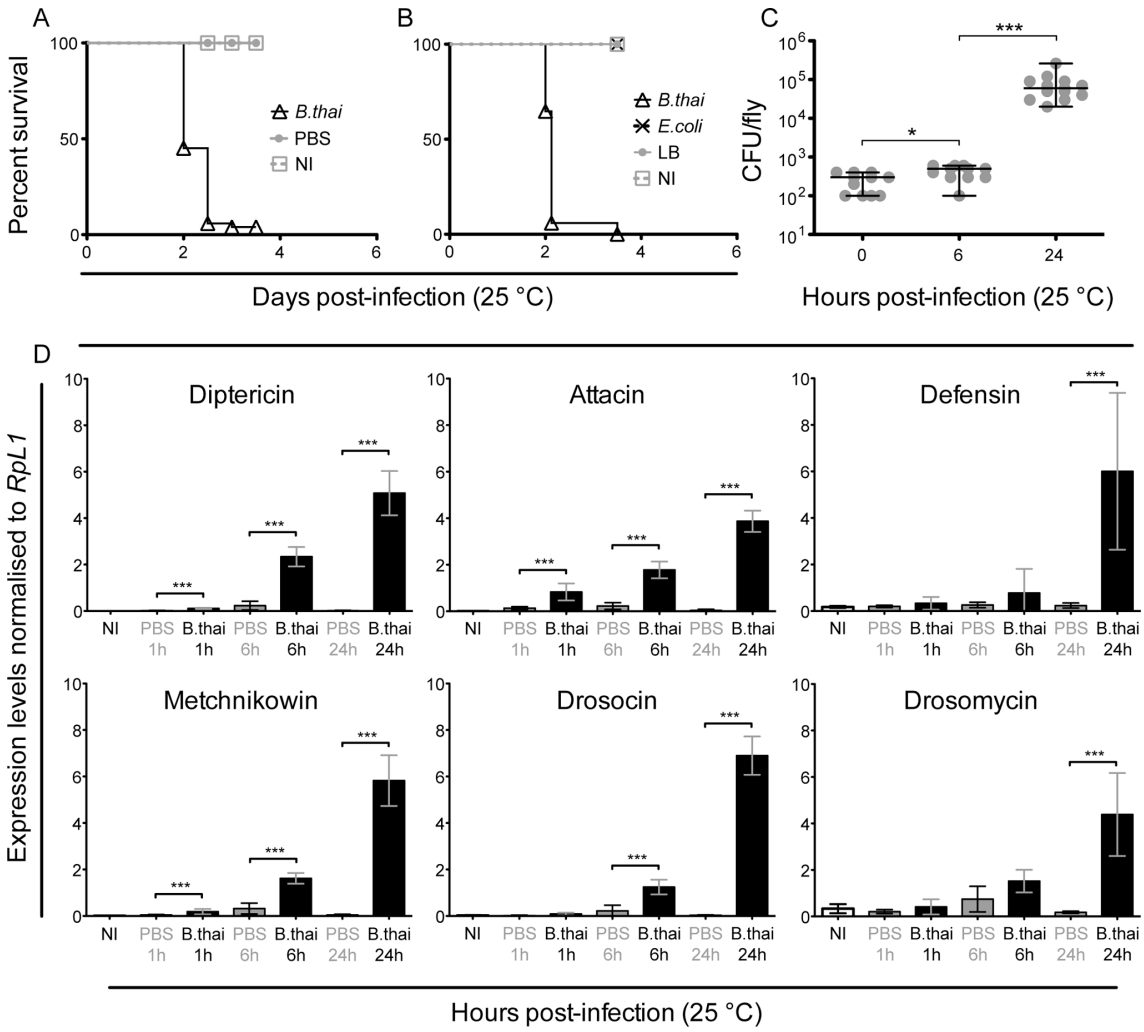


Figure 6.1 ***B. thailandensis*** **infection kills WT male** ***D. melanogaster*****, survives and grows in the host. A.)** *Oregon R* males were infected with WT *B. thailandensis* (*B. thai*) and died within 3.5 days of infection. Survival data was pooled from 3 independent experiments (n = min. 51 males per condition). Bacteria were injected at OD600 = 0.01, -(approximately 250 CFU per fly). Mock-infected (PBS) controls were alive for the duration of this experiment. **B.)** *E. coli*, also a Gram-negative bacterium, was used as a control, and did not kill WT *D. melanogaster*. *E. coli* was injected at OD600 of 1.0; n = min 16 males per condition. **C.)** *B. thailandensis* survived and multiplied inside infected flies. The data is based on 2 independent experiments (n = min. 11 males per time point). *B. thailandensis* was injected at a dose of OD600 = 0.01. Samples were collected at 0, 6 and 24 h p.i. and bacterial growth determined by plating dilutions of homogenised samples. Colonies were counted 24 h after the homogenate was plated and incubated at 37 °C. Statistical significance of bacterial growth between time points was determined using Mann-Whitney test; * $p < 0.02$ and *** $p < 0.0001$. **D.)** *B. thailandensis* infection induced AMP expression in *D. melanogaster*. Three infection time points were analysed: 1, 6, and 24 h; controls were either mock-infected (PBS) or uninjected (NI). All tested AMPs were without exception significantly induced 24 h after infection. The following AMPs constitute a good representation of a fly immune response: Diptericin, Attacin, Defensin, Metchnikowin, Drosocin, and Drosomycin. Levels of AMP mRNA were determined by qPCR. Statistical significance between levels of AMP expression was determined using Mann-Whitney test (GraphPad Prism); *** $p < 0.001$. Data is based on 1 experiment, n = 7 males per condition; error bars represent SD.

6.2.2. *B. thailandensis* infection induces strong expression of *D. melanogaster* AMPs

Antimicrobial peptide expression constitutes the humoral arm of the fly immune system. AMPs are secreted bactericidal peptides, directly toxic to bacteria, whose expression is induced by infection: for example, fly Defensin is induced in response to septic injury with *E. coli* (Nehme *et al*., 2011). Some bacteria can be resistant to AMPs; for example, *in vitro*, *B. pseudomallei* is resistant to the human antimicrobial peptide *defensin* HNP-1 (Jones *et al*., 1996). Moreover, some bacterial species, e.g. *M. marinum*, do not induce or actively prevent induction of AMPs (Dionne *et al*., 2003).

B. thailandensis strongly induces AMPs in *Drosophila* [Figure 6.1 D]. Despite the strong induction, bacteria proliferate and infected flies die rapidly. We further investigated this by infecting flies carrying loss-of-function mutations in the two primary immune-inducible NF-kB family members in the fly (*Dif; Rel* double mutants); these animals are incapable of producing antimicrobial peptides in response to immune challenge (Hedengren *et al*., 1999; Rutschmann *et al*., 2000). *Dif; Rel* double mutants exhibited either a very mild increase in susceptibility, or no increase at all, when infected with *B thailandensis* at 25 °C [Figure 6.2]. Thus, we conclude that *Drosophila* AMPs are unable to kill *B. thailandensis*. These results could reflect AMP resistance, as previously observed with *B. pseudomallei*, or could be due to bacteria being physically segregated from AMPs (Jones *et al*., 1996).

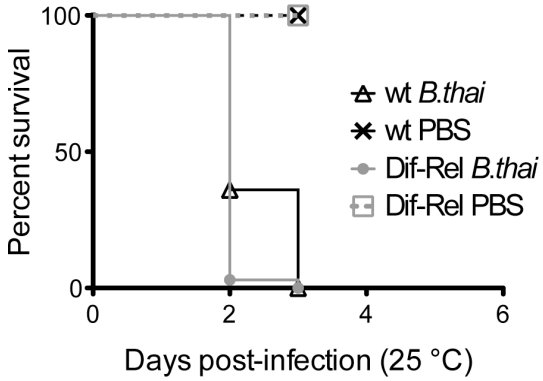


Figure 6.2 **Survival of immunocompromised *Dif; Rel* mutant is comparable to WT animals.** The Toll and Imd pathway double mutant, *Dif; Rel*, was infected with WT *B. thailandensis*, kept at 25 °C, and survival was monitored twice a day. The mutant survival was not significantly different from that of WT male *D. melanogaster*. Data is based on two independent experiments; n = min. 25 flies per condition, except for PBS-injected *Dif; Rel* mutants – 1 experiment, n = 11.

6.2.3. Temperature effect on survival of infected flies and on bacterial growth

We next investigated the role of temperature in this infection. Previous experiments had shown that the distantly-related *Burkholderia cepacia* was capable of killing flies at 18 °C (Schneider *et al*., 2007). We observed that the *B. thailandensis* infection was dramatically slowed when shifted to 18 °C: median survival time increases from 2 days at 25 °C to 20 days at 18 °C [Figure 6.3 A]. This was accompanied by a dramatic increase in bacterial doubling time. Intriguingly, flies could be infected and maintained at 18 °C, with bacterial numbers stable or only very slowly increasing; when these animals were shifted to 25 °C, the infection switched from chronic to acute, with bacterial numbers rapidly increasing and causing the death of the host within one or two days [Figure 6.3 B].

In order to determine whether this was due to effects on the interaction of the host and microbe, or was due simply to changes in microbial metabolic rate, we infected immunocompromised *Dif; Rel* mutants and examined survival after infection. In contrast to the effect seen at 25 °C, *Dif; Rel* mutants infected at 18 °C died much faster than wild-type flies (median survival time = 8 days) [Figure 6.3 C]. The changes in survival time seen at different temperatures are thus caused, at least in part, by changes in the interaction between the bacterium and the host immune response.

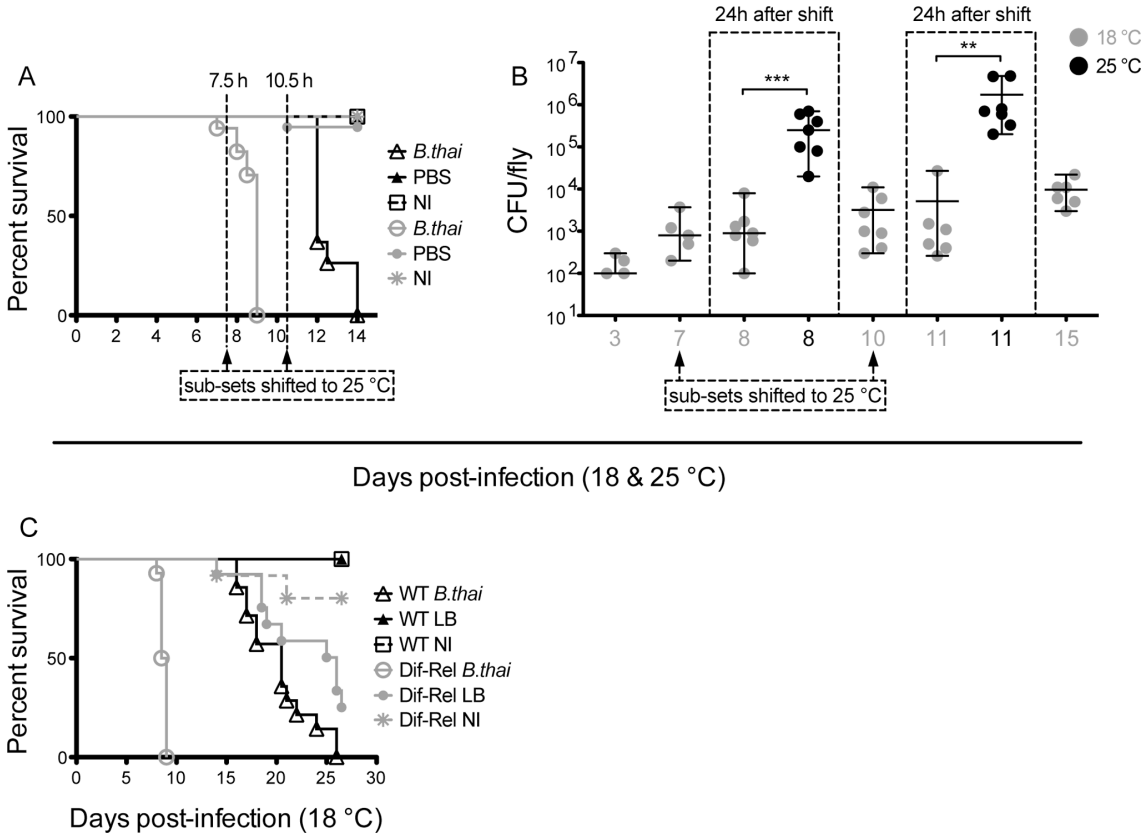


Figure 6.3 ***B. thailandensis*** **growth at 18 °C is slower than at 25 °C. A.)** Infected and control flies were kept at 18 °C, and subgroups were shifted to 25 °C at time points 7.5 and 10.5 days after infection. Dead flies were counted twice a day. The result indicates that bacteria recovered at 25 °C, and killed the flies fast. **B.)** *B. thailandensis* was injected at an initial dose of OD600 = 0.01. Flies were kept at 18 °C (grey) and shifted to 25 °C (black) at time points 7 and 10 days p.i. Subsets of equally treated flies were kept at 18 °C as controls (grey). Samples were homogenised 24 h after shifting from 18 to 25 °C to determine the growth of bacteria inside the flies. Samples were analysed at time points 3, 7, 8, 10, 11, and 15 days p.i. Bacterial growth was determined by plating dilutions of homogenised infected and control flies in PBS. Plated bacteria were left at 37 °C for 24 h, when bacterial colonies were counted. Data is based on one experiments; n = 7 flies. Statistical significance of bacterial growth was determined using Mann-Whitney test (GraphPad Prism); ** $p < 0.002$, *** $p < 0.001$. Y-axis = log10. **C.)** Simultaneous Toll and Imd pathway mutation (Dif; Rel) decreases survival of B. thai-infected flies at 18 °C, not at 25 °C. Dif; Rel loss-of-function double mutant flies were infected with WT *B. thailandensis* as usual, but were kept at 18 °C. Under these conditions, the mutants were significantly shorter-lived. Statistical significance between the survival curves of infected WT and mutant flies was determined using Log-rank analysis (Mantel-Cox); $p < 0.0001$.

6.2.4. Sterile *B. thailandensis*-conditioned medium is lethal in the fly

B. pseudomallei causes pathology in part by the production of exotoxins (Haase *et al*., 1997; Häussler *et al*., 1998). In order to see whether some exotoxin might account for some or all of the lethality observed in this infection, we injected flies with sterile spent media in which *B thailandensi*s had previously grown. *B. thailandensis* was grown overnight in LB at 37 °C. The culture was spun at 2400 x g for 4 minutes; supernatant was removed into a new tube and sterile-filtered using a 0.2 μm filter. To ensure that the sterile conditioned medium (CM) contained no live bacteria, a portion of the same CM that was injected into flies was plated on LB agar and kept at 37 °C for 48 hours; no colonies grew (data not shown). As a control for this set of experiments, LB was kept overnight at 37 °C alongside the incubating *B. thailandensis* culture, processed precisely the same way as the bacterial culture, and used for mock-infections. Portion of the sterile-filtered LB was also plated to prove that it had not been contaminated; no colonies grew at 37 °C in 48 hours . When the sterile-filtered *B. thailandensis*-conditioned medium was injected into WT flies, it killed them nearly as rapidly as live *B. thailandensis* [Figure 6.4 A]. *E. coli*-conditioned medium was used as a negative control; no deaths resulted from this infection [Figure 6.8 E].

Despite being lethal to WT flies, *B. thailandensis*-conditioned medium does not induce a systemic immune response: *D. melanogaster* AMPs Drosocin and Attacin, which were strongly induced by infection with live bacteria, were not induced [Figure 6.4 B].

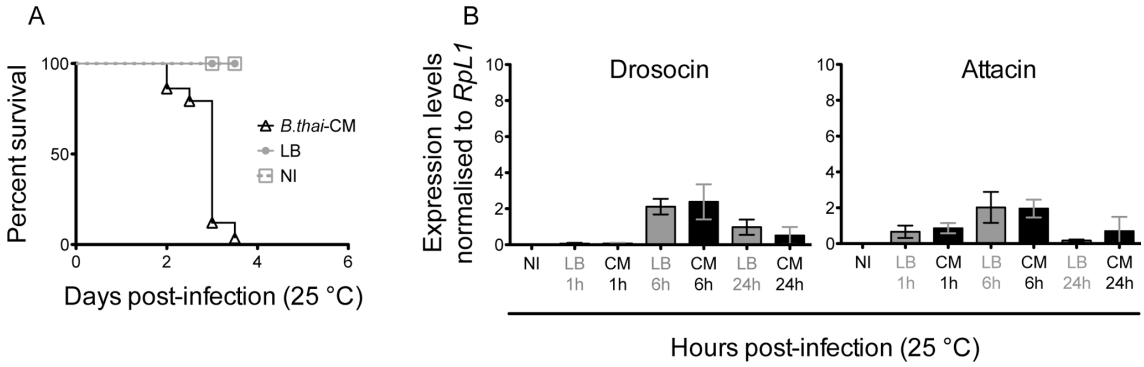


Figure 6.4 **Sterile *B. thai*-conditioned medium is almost as virulent as live bacteria. A.)** *Oregon-R* males injected with sterile-filtered *B. thailandensis*-conditioned medium (CM) died as rapidly as those infected with live *B. thailandensis*. Mock-infected (LB) and uninjected (NI) controls continued to live at least for the duration of this experiment; data is based on 3 independent experiments, n = min 56 males per condition. **B.)** Fly antimicrobial peptides, Drosocin and Attacin, were not induced by *B. thailandensis*-conditioned medium. The levels of AMP mRNA were determined by qPCR; data is based on 1 experiment, n = 7 males per condition; error bars represent SD.

6.2.5. *B. thailandensis E264* type-3 and type-6 secretion systems do not play a role in virulence to *D. melanogaster*

Schwarz and colleagues observed that *B. thailandensis* lacking Type VI secretion system number 5 (ΔT6SS-5) had reduced virulence in mice, while T6SS-1 was important in *B. thailandensis* survival in competition with other Gram-negative bacteria, such as *Pseudomonas putida* and *Serratia proteamaculans* (Schwarz *et al*., 2010). In flies, we found that a *B. thailandensis* mutant lacking all five Type VI secretion systems, ΔT6SS-(1-6), exhibited wild-type virulence at 25 °C, as was also true of the single ΔT6SS-5 mutant: in each case, over 94% of infected flies died within 2.5 days of infection [Figure 6.5 A and B].

We also tested the Bsa Type III secretion system mutant (AH174) and the complemented mutant (AH186) (Haraga *et al*., 2008). The mutation had no effect on the survival of *B. thailandensis*-infected flies at 25 °C [Figure 6.5 C and D]. Controls consisted of *E. coli*-infected flies, PBS-injected flies, and untreated flies. Bacterial growth in $T3SS_{Bsa}$ mutant was not significantly different from WT *B. thailandensis* [Figure 6.5 E].

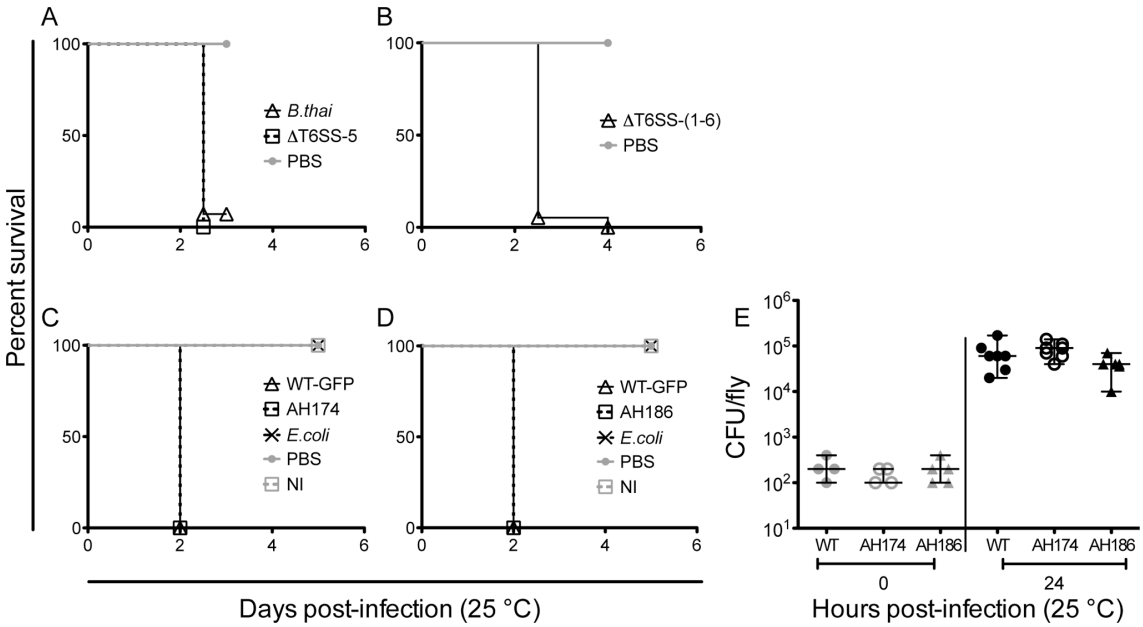


Figure 6.5 **Type III (T3SS) and VI (T6SS) secretion systems are not required for virulence in *Drosophila*. A.)** The *B. thailandensis* mutant lacking only the number 5 T6SS (ΔT6SS-5) was no less pathogenic than WT *B. thailandensis*. **B.)** A bacterial mutant lacking all T6SS [ΔT6SS-(1-6)] was also tested, but no significant difference in survival was noted. **C.)** The Type III secretion system *B. thailandensis* mutant (AH174) was no less pathogenic than WT *B. thailandensis*. **D.)** A complement AH186 mutant appears to cause normal virulence. **E.)** Bacterial growth of the T3SS mutant (AH174), and complement AH186 mutant was measured, but appeared normal. Statistical significance of bacterial growth between time points was determined using Mann-Whitney test.

6.2.6. Food infected with *B. thailandensis E264* kills wild-type flies

In order to examine the effects of oral infection with *B thailandensis*, we inoculated a potato-milk-fructose *Drosophila* food mix with the GFP-expressing strain, AH181 (Haraga *et al*., 2008). Flies transferred onto this food apparently remained healthy for at least 24 hours, but by 48 hours, many flies had died [Figure 6.6 A]. Flies that were surviving at this time were transferred to fresh uninfected food; these animals nonetheless succumbed to the infection. On dissection, GFP-expressing bacteria were clearly present in the gut [Figure 6.6 B and C]; in particular, the crop of these animals tended to be dramatically distended and often contained large amounts of GFP-positive material. Thus, this organism does not require external assistance to circumvent the barrier defenses of the fly. The GFP-labelled *B. thailandensis* was tested in a survival assay to ensure that the lethality of this and the wild-type *Oregon R* (WT) strain was comparable [Figure 6.8 F].

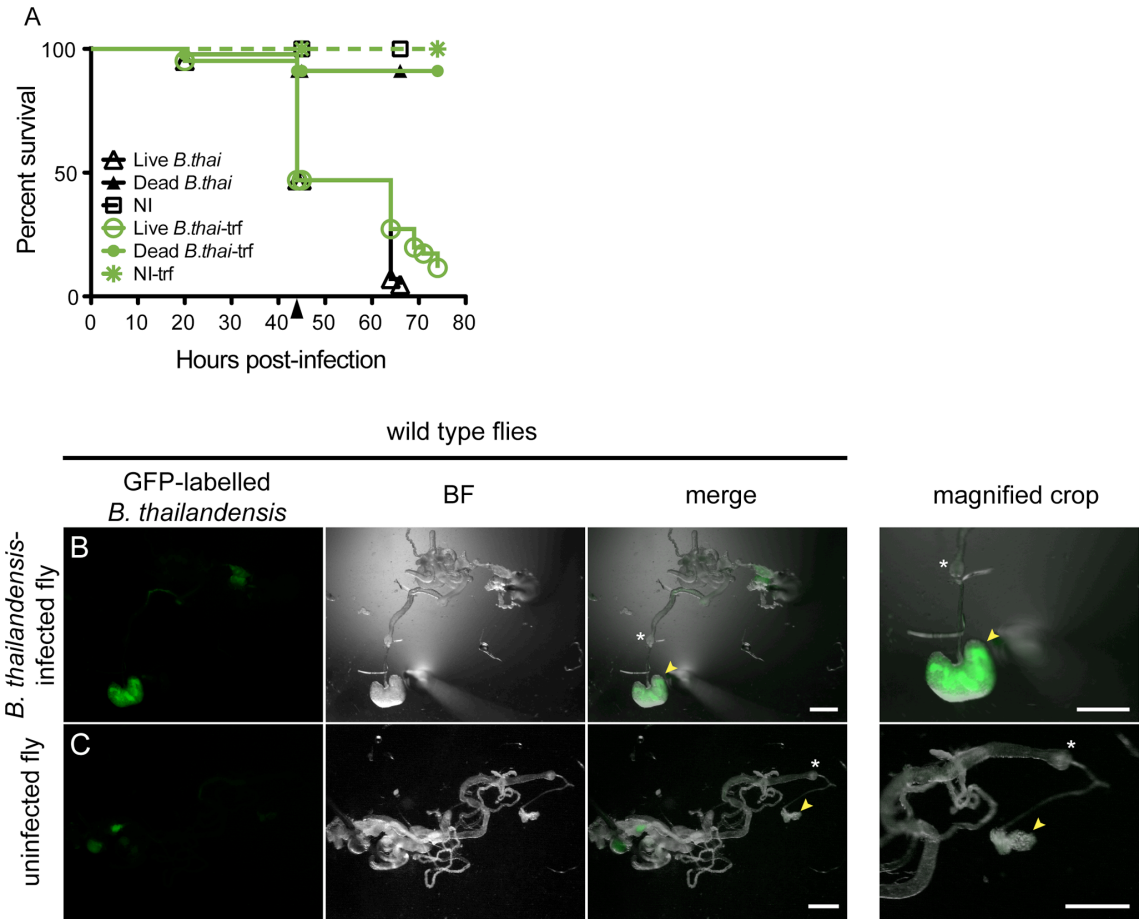


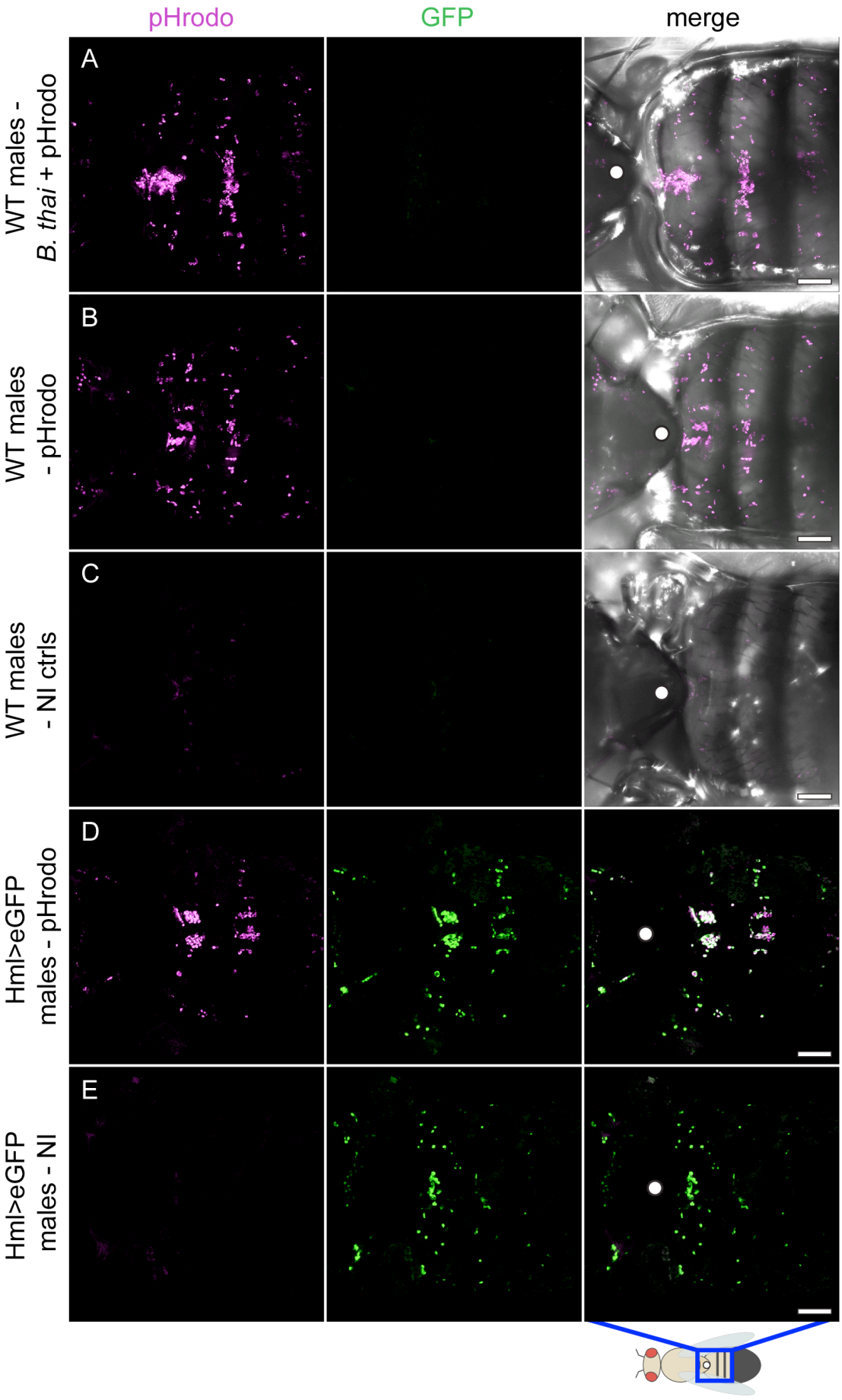
Figure 6.6 **WT flies fed *B. thailandensis*-infected food are killed and have enlarged crop.**
A.) Survival of WT flies on infected food. Flies kept on infected food died within 3.5 days after they were placed on this food. Second set of flies (trf) was kept on infected food and transferred to normal food, free of bacteria, at 44 h (black arrowhead). The survival of the transferred flies was slightly increased in comparison to the non-transferred group, but this difference was not significant. Controls were fed either food containing heat-killed *B. thailandensis* or no bacterium. The survival of the control groups was not affected. Sample size was at least 40 flies per condition. **B.)** Dissected gut of WT male *D. melanogaster* fed food infected with GFP-labelled B. thailandensis. The presence of the bacteria in the crop is confirmed by green fluorescence, which is visible only in the infected flies. **C.)** An uninfected control had smaller crop. The crops of the infected and uninfected flies are shown at a higher magnification [magnified crop]. At least 3 flies were imaged per condition. Yellow arrowheads point to crop; white asterisks mark the proventriculus. Scale bars represent 500 μm.

6.2.7. *D. melanogaster* haemocytes are not destroyed by *B. thailandensis* 24 hours before death

Some infections in *Drosophila* inhibit the bactericidal phagocyte system. To understand what effect *B. thailandensis* has on haemocytes we used pHrodo-labelled *E. coli* BioParticles® (pHrodo). pHrodo is rhodamine-based dye that is conjugated to dead bacteria as a probe for phagocytosis; it is red fluorescent only at a low pH, such as that found in phagocytic vesicles. This fluorogenic feature allows specific imaging of phagocytosis and also, in this case, confirmed that injected pHrodo-labelled bacteria were internalised by haemocytes of *B. thailandensis*-infected *D. melanogaster* approximately 24 h before the host was killed by this infection [Figure 6.7 A]. The obtained data shows that the distribution of pHrodo-containing haemocytes in infected flies is comparable to that of flies injected only with pHrodo, but no bacteria [Figure 6.7 B]. Untreated controls were imaged at the same time as infected flies; no fluorescence was visible, only slight auto-fluorescence was noted [Figure 6.7 C]. All infected and control flies were imaged in a GFP channel. In addition, we used *D. melanogaster* expressing eGFP in a haemocyte-specific manner, *HmlΔGAL4, UAS-2xeGFP*, as a control to show the co-localisation of pHrodo and haemocytes [Figure 6.7 D]; untreated controls were also imaged [Figure 6.7 E].

Based on our results, *B. thailandensis* infection in *D. melanogaster* had not destroyed the phagocytic capabilities of fly haemocytes approximately 24 h before death.

Figure 6.7 ***B. thailandensis*** **infection does not appear to affect phagocytic function of adult plasmatocytes. A.)** To examine the fate of haemocytes in this infection, WT males were infected with WT *B. thailandensis*, and 24 h later injected with pHrodo. Flies were imaged 4 - 5 h after pHrodo injection. The pHrodo beads were localised to haemocytes (magenta). **B.)** WT controls injected with pHrodo only. **C.)** Uninjected controls (NI). **D.)** pHrodo-injected flies expressing eGFP in a haemocyte-specific pattern (*HmlΔGAL4, UAS-2xeGFP*); at least 3 flies were imaged per condition. pHrodo is visible in magenta; co-localisation in white. **E.)** Untreated Hml>eGFP controls. The pattern of phagocytosed pHrodo was consistent with the pattern of haemocytes of NI flies that expressed eGFP in haemocytes. Since the pHrodo dye is bright fluorescent red only in an acidic environment, this result suggests that ~24 h before death, haemocytes of infected flies are functioning and visible (magenta). Scale bars represent 100 μm. The cartoon shows the dorsal side of *D. melanogaster*; the blue rectangle marks the area that was imaged; the white dot marks the notum.


pHrodo
GFP
merge
A
WT males - B. thai + pHrodo
B
WT males - pHrodo
C
WT males - NI ctrls
D
Hml>eGFP males - pHrodo
E
Hml>eGFP males - NI

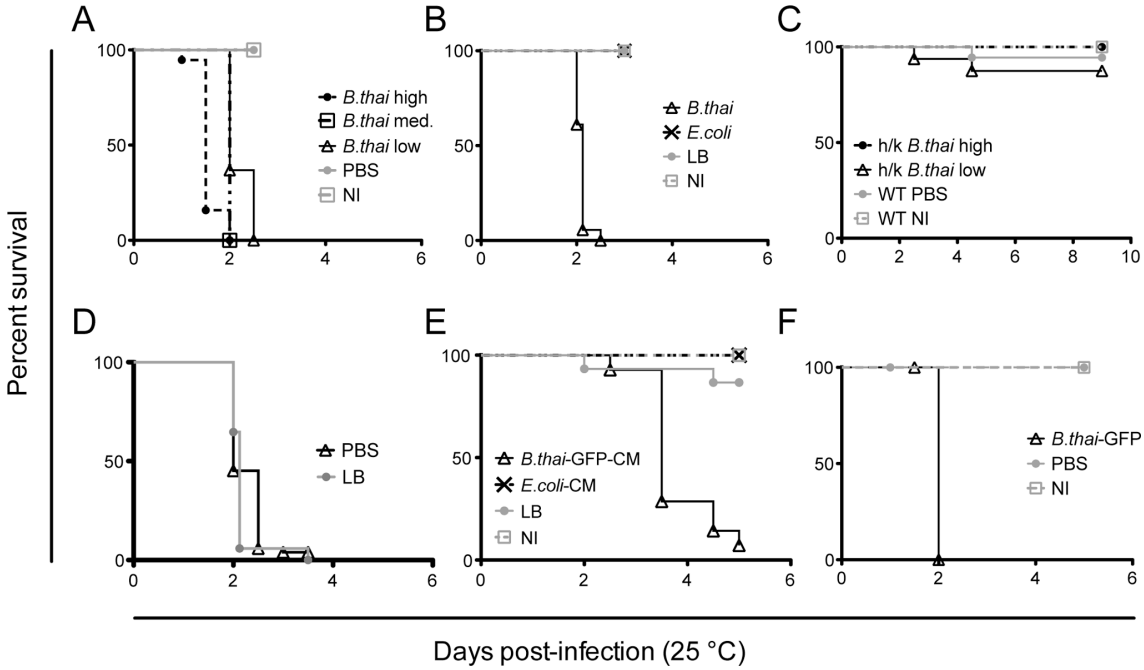


Figure 6.8 Supplementary figures: **A.) Infected *D. melanogaster* was killed in a dose-dependent manner.** The result is based on a single experiment; n = 19 flies per genotype per condition. Three infectious doses were tested: OD600 = 0.01 (low), 0.1 (medium), and 1 (high). Mock-infected (PBS) and untreated (NI) controls were alive for the whole duration of this experiment. **B.)** *B. thailandensis* was as pathogenic in w1118 males as it was in Oregon-R flies [Figure 1A]. *B. thailandensis* bacteria were suspended in LB. *E. coli* was used as a negative control; *E. coli*-infected flies did not die. **C.)** Heat-killed *B. thailandensis* was avirulent in WT males. High dose of *B. thailandensis* was OD600 of 1; low OD600 of 0.01. **D.)** *B. thailandensis* was equally virulent when suspended in its original medium (LB) or resuspended in PBS. **E.)** *E. coli*-conditioned medium (*E.coli*-CM) was not infectious in comparison to that of *B. thailandensis* (*B. thai*-GFP-CM). **F.)** Flies infected with GFP-labelled *B. thailandensis* died within 2 days p.i., which is comparable to infections with non-GFP-labelled *B. thailandensis*.

6.3. Conclusion

In this study, we tested *B. thailandensis* as a potential *D. melanogaster* pathogen and found that the bacterium was highly virulent in the fly. This bacterium is mostly avirulent in humans, but exceptions have been recorded where *B. thailandensis* infection resulted in melioidosis-like symptoms (Glass *et al*., 2006; Lertpatanasuwan *et al*., 1999). *Drosophila* has been shown to be a genetically tractable model in other infections (Brandt *et al*., 2004; D'Argenio *et al*., 2001; Dionne *et al*., 2003; Needham *et al*., 2004).

B. thailandensis survives and multiplies in infected flies. The bacterium grows well at 25 - 37 °C (Brett *et al*., 1997) and when injected into *Drosophila*, it multiplies until the time of the host's death. The lethal dose of *B. thailandensis* is approximately 250 CFU per fly. Growth between 0 h and 6 h post-infection is slow and statistically insignificant; however, the bacterial burden at 24 h post-infection was significantly higher in comparison to that obtained at 6 h p.i.

Although *B. thailandensis* infection induces expression of *Drosophila* AMPs, the bacterium kills its host within 48 hours. This result suggests that *B. thailandensis* may be resistant to AMPs, much as *B. pseudomallei* is resistant to human defensin HNP-1 *in vitro* (Jones *et al*., 1996). In this study *B. pseudomallei*, but not *S. typhimurium* or *E. coli*, was resistant to HNP-1 (Jones *et al*., 1996). Alternatively, the bacteria might be inside cells, shielding them from the effect of the antimicrobial peptides. The phagocytic function of haemocytes was tested in *B. thailandensis*-infected *Drosophila* using pHrodo, and, interestingly, did not appear to be affected by this infection: pHrodo was clearly phagocytosed.

B. thailandensis has proven to be a fast killer of *D. melanogaster* at 25 °C. It is possible that lack of adaptive immunity and good growth conditions for the bacterium cause this infections to be a speedy killer. It is also possible that *B. thailandensis* at optimal conditions causes an "acute" infection and demonstrates resistance to antimicrobial peptides, which are clearly expressed. An immunocompromised *Dif; Rel* double mutant does not die faster as would be expected. Consequently, AMPs might not seem to have much effect. However, when WT and *Dif; Rel* mutants were subjected to this infection at a lower temperature of 18 °C a significant difference in survival emerged; the *Dif; Rel* mutant was shorter-lived. This data suggests that the bacteria could be either to some extent protected from the effect of AMPs or are indeed resistant to the bactericidal proteins.

Another part of our study looked at the potential function of Type III and VI secretion systems. These systems were previously shown to be implicated in *B. thailandensis* virulence; $T3SS_{Bsa}$ in mice (Haraga *et al*., 2008), and the number five of the T6SS also in mice (Schwarz *et al*., 2010). In the fly, $T3SS_{Bsa}$ does not have attenuated phenotype, nor does the T6SS-5. In the future, this infection should be tested at 18 °C.

Heat-inactivation of *B. thailandensis* renders it avirulent in *D. melanogaster*. However, a sterile *B. thailandensis*-conditioned medium, completely free of live bacteria, proved to be as pathogenic in the fly as live bacteria. This result suggests that *B. thailandensis* secretes an exotoxin. The exotoxin might share similarity to toxins secreted by *B. pseudomallei* (Haase *et al*., 1997; Häussler *et al*., 1998). Although it is interesting that the *B. thailandensis* 'toxin' alone kills, the bacterial culture washed and resuspended in PBS, and thus free of the 'toxin', kills faster in comparison with the

sterile bacteria-conditioned medium, implying that the exotoxin present in spent media cannot be the sole effector of bacterial pathogenicity.

Next, we imaged infected flies, but the anatomical location of *B. thailandensis* was inconsistent [data not shown]. However, following an oral infection with GFP-labelled *B. thailandensis*, we obtained images of dissected fly crops. The crops of infected flies were distended and clearly fluorescent in comparison to controls. These data suggest that the bacterium is capable of killing via oral infection, and grow inside the fly digestive system.

The aim of this study was to establish *D. melanogaster* as a model for the study of host-pathogen interaction with *B. thailandensis*. Since the fruit fly immune response is elicited only via the innate immune system, it could prove to be a useful model for the study of the role of innate immunity in melioidosis.

Chapter 7. DISCUSSION

The data reported in this thesis was obtained from several projects that were not necessarily related to each other; for example, *B. thailandensis* infections of the fly were not connected with those of *M. marinum*. Therefore, this chapter is divided into sections according to individual projects.

7.1. The role of *shf* in *M. marinum* infection in the fly

Initially, I examined the possible involvement of the *shifted* gene in *M. marinum*-induced pathology in *D. melanogaster*. Induction of the *shf* gene and immune responses in S2 *Drosophila* tissue culture cells to different bacteria - *M. smegmatis, M. luteus, E. coli*, and *M. marinum* – were also studied. *In vitro* experiments were done in time when facilities were not ready for *in vivo* survival assays. Results from *in vitro* experiments were not completed and conclusions cannot be drawn from them due to small sample sizes.

In vivo experiments involved survival assays to confirm preliminary data, and further experiments in an attempt to analyse *shf* expression by qRT-PCR. Flies with ubiquitous and tissue-specific knockdown of *shf* were tested for difference in survival following *M. marinum* infection. The survival experiments were repeated several times, but they did not confirm preliminary data (Dionne, unpublished observations). As a control, the *shf.IR* was driven by the ubiquitous driver *Tubulin* or *daughterless*, and the knockdown was thus confirmed in untreated flies using qRT-PCR. The discrepancy between the preliminary and new survival data was most likely caused by a difference

in fly food composition. There might be another explanation, but the above-mentioned reason is plausible given the fact that the life span of *D. melanogaster* and other animals is affected by food intake (Fontana *et al*., 2010). The progress of infection could be equally affected and the host's resistance to bacteria could be altered by different food. Before any progress to study the function of *shf* in mycobacterial infection is attempted, a thorough study of richness of food should be undertaken to establish the effect of different fat and/or sugar content on the *Drosophila* immune system.

7.2. *In vivo* imaging of infection in immobilised *D. melanogaster*

The goal of this study was essentially to test an immobilising technique that would allow imaging of infected flies *in vivo* at different time points during infection. After a new immobilising technique using cyanoacrylate-based glue worked, I attempted to obtain time-lapse movies of the progress of phagocytosis of DsRed *M. marinum* in flies expressing eGFP in haemocytes. I did not achieve this, perhaps due to a lack of skill and experience at the start of this particular part of this work. However, the immobilising technique works well and some colleagues found it useful (Clark *et al*., 2011).

In parallel to my attempts at imaging the progress of phagocytosis, I attempted and succeeded in imaging the progress of infection, which was documented at no less than two different time points, 24 hours apart. Progressing *M. marinum* infection appears to deplete haemocytes or at least GFP^{+ve} haemocytes. To find out if *M. marinum*-infected haemocytes undergo apoptosis, similar to mammalian macrophages infected with *M. tuberculosis* (Schaible *et al*., 2003), flies with anti-apoptotic protein p35 overexpression specifically in haemocytes were tested. The results suggest that the survival of *D. melanogaster* during *M. marinum* infection is not affected by p35 overexpression and the infected flies survive as long as controls. pHrodo, a pH-sensitive

rhodamine-based dye conjugated to dead *E. coli*, co-localises with GFP^{+ve} haemocytes in infected flies. However, it remains to be confirmed whether all haemocytes are depleted during *M. marinum* infection or only those expressing GFP under the control of *Hemolectin*.

7.3. Screen of haemocyte-specific gene knockdown in relation to *M. marinum* infection

The goal of this study was to test haemocyte-specific knockdown of genes directly or indirectly involved in phagocytosis in order to identify a potential *M. marinum*-specific receptor in the fly. This screen did not result in any clear-cut conclusions in this regard, though several interesting observations were made.

The scavenger receptor Peste is the only known mycobacterium-specific receptor *in vitro* (Philips *et al*., 2005). Haemocyte-specific silencing of the *peste* gene using RNAi *in vivo* did not show any significant difference in survival during *M. marinum* infection. This work did not confirm the *in vitro* data of peste specificity, nor did it establish novel *M. marinum*-specific receptor(s). It is possible that *M. marinum* phagocytosis in the fly does not depend on a single receptor, and thus it might be necessary to test double knockdown, and where possible loss-of-function mutants.

A preliminary imaging screen using fluorescent microscopy did not result in good quality pictures, but this screen was intended to be swift to reveal clear defects or advantages of phagocytosis in haemocyte-specific knockdown of various genes of interest. However, a more focused screen using confocal microscopy together with qRT-PCR analysis revealed clear phenotypes in several knockdowns. For example, the haemocyte-specific knockdown of *Snmp1*, *CG3829*, *CG1887*, *CG2736*, *CG10345*, *CG7227* or *SR-CIV* rendered flies less susceptible to *M. marinum* infection then

controls. This phenotype is likely to be caused either due to altered function of haemocytes or due to a different phenomenon occurring in the infected knockdowns. Apart from the *Snmp1* and *nimC3* knockdowns, no haemocyte phenotype was observed during the fast preliminary imaging screen.

The phenotypes of *nimC3* knockdown flies is both interesting and confusing due to the following reasons: 1. preliminary screen revealed lower number of haemocytes in untreated controls (data not shown); 2. survival of these *nimC3* knockdowns was not affected in *M. marinum* in comparison to controls; 3. the phenotype observed during the preliminary screen did not repeat during a more focused screen using confocal microscopy; 4. *Hml* expression levels in infected and control flies were comparable to those of controls; 5. *M. marinum* burden was significantly higher in these knockdowns; 6. AMP expression was comparable to that of infected controls apart for Metchnikowin expression, which was diminished.

Metchnikowin is classed as an AMP with antifungal and antibacterial activity that is regulated by the Toll and Imd pathways (Levashina *et al*., 1995; Levashina *et al*., 1998). At the moment, it is only possible to speculate why and how the expression of Metchnikowin is diminished by *M. marinum* infection in *Drosophila*. It may be that Metchnikowin induction during infection requires signalling specifically between Hml^{+ve} haemocytes, which are clearly affected by *M. marinum* infection [Figure 5.9], and the fat body.

Altogether the data suggests that some genes coding for phagocytic or scavenger receptors could be crucial in *M. marinum* infection, but this needs to be confirmed using another haemocyte-specific driver.

7.4. *B. thailandensis* causes lethal infection in the fly

In this study, *B. thailandensis* was tested as a potential *D. melanogaster* pathogen and found that the bacterium was highly virulent in the fly. This bacterium is mostly avirulent in humans, but exceptions have been recorded where *B. thailandensis* infection resulted in melioidosis-like symptoms (Glass *et al*., 2006; Lertpatanasuwan *et al*., 1999). *Drosophila* has been shown to be a genetically tractable model in other infections (Brandt *et al*., 2004; D'Argenio *et al*., 2001; Dionne *et al*., 2003; Needham *et al*., 2004).

Although *B. thailandensis* infection induces expression of *Drosophila* AMPs, the bacterium kills its host within 48 hours. This result suggests that *B. thailandensis* may be resistant to AMPs, much as *B. pseudomallei* is resistant to human defensin HNP-1 *in vitro* (Jones *et al*., 1996). In this study *B. pseudomallei*, but not *S. typhimurium* or *E. coli*, was resistant to HNP-1 (Jones *et al*., 1996). Alternatively, the bacteria might be inside cells, shielding them from the effect of the antimicrobial peptides. The phagocytic function of haemocytes was tested in *B. thailandensis*-infected *Drosophila* using pHrodo, and, interestingly, did not appear to be affected by this infection: pHrodo was clearly phagocytosed.

B. thailandensis has proven to be a fast killer of *D. melanogaster* at 25 °C. It is possible that lack of adaptive immunity and good growth conditions for the bacterium cause this infections to be a speedy killer. It is also possible that *B. thailandensis* at optimal conditions causes an “acute” infection and demonstrates resistance to antimicrobial peptides, which are clearly expressed. An immunocompromised *Dif; Rel* double mutant does not die faster as would be expected. Consequently, AMPs might not seem to have much effect. However, when WT and *Dif; Rel* mutants were subjected to this infection at a lower temperature of 18 °C a significant difference in survival

emerged; the *Dif; Rel* mutant was shorter-lived. This data suggests that the bacteria could be either to some extent protected from the effect of AMPs or are indeed resistant to the bactericidal proteins.

Another part of this study looked at the potential function of Type III and VI secretion systems. These system were previously shown to be implicated in *B. thailandensis* virulence; $T3SS_{Bsa}$ in mice (Haraga *et al*., 2008), and the number five of the T6SS also in mice (Schwarz *et al*., 2010). In the fly, $T3SS_{Bsa}$ does not have attenuated phenotype, nor does the T6SS-5. In the future, this infection should be tested at 18 °C.

Heat-inactivation of *B. thailandensis* renders it avirulent in *D. melanogaster*. However, a sterile *B. thailandensis*-conditioned medium, completely free of live bacteria, proved to be nearly as pathogenic in the fly as live bacteria. This result suggests that *B. thailandensis* secretes an exotoxin. The exotoxin might share similarity to toxins secreted by *B. pseudomallei* (Cruz-Migoni *et al*., 2011; Haase *et al*., 1997; Häussler *et al*., 1998). Although it is interesting that the *B. thailandensis* 'toxin' alone kills, the bacterial culture washed and resuspended in PBS, and thus free of the 'toxin', kills faster in comparison with the sterile bacteria-conditioned medium, implying that the exotoxin present in spent media cannot be the sole effector of bacterial pathogenicity. However, this result strongly suggests that *B. thailandensis* produces a toxin that plays a major role in its pathogenesis in the fly.

Next, I imaged infected flies *in vivo*, but the anatomical location of *B. thailandensis* was inconsistent. In a first set of images, the GFP-labelled bacteria were detected in a specific location [data not shown]. However, following an oral infection with GFP-labelled *B. thailandensis*, I obtained images of dissected fly crops. The crops of infected flies were distended and clearly fluorescent in comparison to controls. These

data suggest that the bacterium is capable of killing via oral infection, and grow inside the fly digestive system.

Together the data suggests that *B. thailandensis* kills the fly probably in a combination of bacterial resistance to AMPs and as a result the immune system of the fly might compensate by expressing all the AMPs, even those that are usually fungus-specific, such as Drosomycin. This AMP overload might have a detrimental effect in itself. Then the production of *B. thailandensis* "toxin" obviously contributes to the bacterial pathogenesis in the fly.

It is difficult to make a direct comparison between the pathology of *B. pseudomallei* in mammals and that of *B. thailandensis* in *Drosophila*. People who are especially in danger of contracting melioidosis in endemic areas are those with pre-existing chronic condition, such as diabetes mellitus (Cheng and Currie, 2005; Hassan *et al*., 2010; Limmathurotsakul *et al*., 2010). I have used *Drosophilae* that were expressing a dominant negative form of the insulin receptor to simulate insulin resistance in the fly. Upon infection, *B. thailandensis* burden was measured, and the result was comparable to that of controls (data not shown). Perhaps survival of these flies during *B. thailandensis* infection would have been altered, but at this stage, parallels cannot be drawn with pathology of this particular condition.

Although the molecular mechanism of *B. thailandensis* pathogenicity in the fly is not yet clear, data in this thesis provides strong evidence that *B. thailandensis* "toxin" is important in this pathology. This is supported by the fact that a cytotoxin has recently been described in *B. pseudomallei* (Cruz-Migoni *et al*., 2011).

A recent study reported that *Burkholderia* species, such as SFA1 [AB232333], are gut symbionts in the agricultural pest *Riptortus pedestris*. These bacteria are capable of detoxifying the insecticide fenitrothion by degrading it to a less potent compound, and

thus making the host resistant to the use this insecticide (Kikuchi *et al*., 2012). Although the *Burkholderia* species SFA1 [AB232333] are not pathogenic to their host, it appears that others, such as *Burkholderia cenocepacia*, can gain antibiotic resistance in a similar manner, by degrading antibiotics (Sass *et al*., 2011).

The aim of this study was to establish *D. melanogaster* as a model for the study of host-pathogen interaction with *B. thailandensis*. Since the fruit fly immune response is elicited only via the innate immune system, it could prove to be a useful model for the study of the role of innate immunity in melioidosis.

7.5. Future significance

The advantages of using this particular model system are as follows: fly disease is similar to human disease in some aspects; advanced *Drosophila* genetics enable easy identification of the genes important in disease. Ultimately, predictions that come from fruit flies can then be tested in human populations. The underlying motivation of all the work reported in this thesis was to elucidate the underlying molecular signalling in *M. marinum* infected flies, to reveal an *M. marinum*-specific phagocytic receptor, and to test if *B. thailandensis* infection in the fly could model a more serious disease in people, melioidosis. Consequently, this knowledge could be applied on a higher level to infections in humans, such as tuberculosis or melioidosis.

INDEX OF FIGURES

INDEX OF TABLES

BIBLIOGRAPHY

Abrams, J.M., Lux, A., Steller, H., Krieger, M., 1992. Macrophages in Drosophila embryos and L2 cells exhibit scavenger receptor-mediated endocytosis. Proc. Natl. Acad. Sci. U. S. A. 89, 10375-10379.

Agaisse, H., Petersen, U., Boutros, M., Mathey-Prevot, B., Perrimon, N., 2003. Signaling Role of Hemocytes in Drosophila JAK/STAT-Dependent Response to Septic Injury. Dev. Cell 5, 441–450.

Akbar, M.A., Tracy, C., Kahr, W.H.A., Kräimer, H., 2011. The full-of-bacteria gene is required for phagosome maturation during immune defense in Drosophila. J. Cell Biol. 192, 383–390.

Åsling, B., Dushay, M.S., Hultmark, D., 1995. Identification of early genes in the Drosophila immune response by PCR-based differential display: the Attacin A gene and the evolution of attacin-like proteins. Insect Biochem. Mol. Biol. 25, 511–518.

Bangs, P., White, K., 2000. Regulation and execution of apoptosis during Drosophila development. Dev. Dyn. 218, 68-79.

Bardet, P., Kolahgar, G., Mynett, A., Miguel-Aliaga, I., 2008. A fluorescent reporter of caspase activity for live imaging. PNAS 105, 13901–13905.

Barker, J.R., Chong, A., Wehrly, T.D., Yu, J.-J., Rodriguez, S.A., Liu, J., Celli, J., Arulanandam, B.P., Klose, K.E., 2009. The Francisella tularensis pathogenicity island encodes a secretion system that is required for phagosome escape and virulence. Mol. Microbiol. 74, 1459-1470.

Barker, L.P., George, K.M., Falkow, S., Small, P.L., 1997. Differential trafficking of live and dead Mycobacterium marinum organisms in macrophages. Infect. Immun. 65, 1497-1504.

Bejsovec, A., Arias, A.M., 1991. Roles of wingless in patterning the larval epidermis of Drosophila. Development 113, 471-485.

Boman, H.G., Nilsson, I., Rasmuson, B., 1972. Inducible antibacterial defence system in Drosophila. Nature 237, 232-235.

Boman, H.G., Nilsson-Faye, I., Paul, K., Rasmuson, T., Jr., 1974. Insect Immunity I. Characteristics of an Inducible Cell-Free Antibacterial Reaction in Hemolymph of Samia cynthia Pupae. Infect. Immun. 10, 136-145.

Boutros, M., Agaisse, H., Perrimon, N., 2002. Sequential activation of signaling pathways during innate immune responses in Drosophila. Dev. Cell 3, 711-722.

Boutros, M., Kiger, A.A., Armknecht, S., Kerr, K., Hild, M., Koch, B., Haas, S.A., Paro, R., Perrimon, N., 2004. Genome-Wide RNAi Analysis of Growth and Viability in Drosophila Cells. Science 303, 832–835.

Brand, A.H., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401-415.

Brandt, S.M., Dionne, M.S., Khush, R.S., Pham, L.N., Vigdal, T.J., Schneider, D.S., 2004. Secreted Bacterial Effectors and Host-Produced Eiger/TNF Drive Death in a Salmonella-Infected Fruit Fly. PLoS Biol. 2, e418.

Brett, P.J., DeShazer, D., Woods, D.E., 1997. Characterization of Burkholderia pseudomallei and Burkholderia pseudomallei-like strains. Epidemiol. Infect. 118, 137-148.

Brett, P.J., DeShazer, D., Woods, D.E., 1998. Burkholderia thailandensis sp. nov., a Burkholderia pseudomallei-like species. Int. J. Syst. Bacteriol. 48, 317-320.

Bridges, C.B., 1916a. Non-Disjunction as Proof of the Chromosome Theory of Heredity. Genetics 1, 1-52.

Bridges, C.B., 1916b. Non-Disjunction as Proof of the Chromosome Theory of Heredity (Concluded). Genetics 1, 107-163.

Bridges, C.B., 1935. Salivary chromosome maps. J. Hered. 26, 60–64.

Brightbill, H.D., Libraty, D.H., Krutzik, S.R., Yang, R.B., 1999. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. Science 285, 732–736.

Brogden, K.A., 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nature Reviews Microbiology 3, 238-250.

Bulet, P., Dimarcq, J.L., Hetru, C., Lagueux, M., Charlet, M., Hegy, G., Van Dorsselaer, A., Hoffmann, J.A., 1993. A novel inducible antibacterial peptide of Drosophila carries an O-glycosylated substitution. J. Biol. Chem. 268, 14893-14897.

Cameron, G.R., 1934. Inflammation in the caterpillars of Lepidoptera. J. Pathol. Bacteriol. 38, 441–466.

Capdevila, J., Guerrero, I., 1994. Targeted expression of the signaling molecule decapentaplegic induces pattern duplications and growth alterations in Drosophila wings. EMBO J. 13, 4459-4468.

Carlsson, A., Engström, P., Palva, E.T., Bennich, H., 1991. Attacin, an antibacterial protein from Hyalophora cecropia, inhibits synthesis of outer membrane proteins in Escherichia coli by interfering with omp gene transcription. Infect. Immun. 59, 3040-3045.

Castonguay-Vanier, J., Vial, L., Tremblay, J., Déziel, E., 2010. Drosophila melanogaster as a model host for the Burkholderia cepacia complex. PLoS ONE 5, e11467.

Chaowagul, W., Suputtamongkol, Y., Dance, D.A., Rajchanuvong, A., Pattara-arechachai, J., White, N.J., 1993. Relapse in melioidosis: incidence and risk factors. J. Infect. Dis. 168, 1181-1185.

Chaowagul, W., White, N.J., Dance, D.A., Wattanagoon, Y., Naigowit, P., Davis, T.M., Looareesuwan, S., Pitakwatchara, N., 1989. Melioidosis: a major cause of community-acquired septicemia in northeastern Thailand. J. Infect. Dis. 159, 890-899.

Cheng, A.C., Currie, B.J., 2005. Melioidosis: epidemiology, pathophysiology, and management. Clin. Microbiol. Rev. 18, 383-416.

Cho, Y., Griswold, A., Campbell, C., Min, K.-T., 2005. Individual histone deacetylases in Drosophila modulate transcription of distinct genes. Genomics 86, 606-617.

Choe, K., Werner, T., Stöven, S., Hultmark, D., 2002. Requirement for a Peptidoglycan recognition protein (PGRP) in Relish activation and antibacterial immune responses in Drosophila. Science 296, 359–362.

Chung, Y.-S.A., Kocks, C., 2011. Recognition of pathogenic microbes by the Drosophila phagocytic pattern recognition receptor eater. J. Biol. Chem. 286, 26524–26532.

Clark, H.F., Shepard, C.C., 1963. Effect of environmental temperatures on infection with Mycobacterium marinum (Balnei) of mice and a number of poikilothermic species. J. Bacteriol. 86, 1057-1069.

Clark, R.I., Woodcock, K.J., Geissmann, F., Trouillet, C., Dionne, M.S., 2011. Multiple TGF-β superfamily signals modulate the adult Drosophila immune response. Curr. Biol. 21, 1672-1677.

Clay, H., Davis, J.M., Beery, D., Huttenlocher, A., Lyons, S.E., Ramakrishnan, L., 2007. Dichotomous role of the macrophage in early Mycobacterium marinum infection of the zebrafish. Cell Host & Microbe 2, 29-39.

Clemens, D.L., Horwitz, M.A., 1995. Characterization of the Mycobacterium tuberculosis phagosome and evidence that phagosomal maturation is inhibited. J. Exp. Med. 181, 257-270.

Clemens, J.C., Worby, C.A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B.A., Dixon, J.E., 2000. Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. PNAS 97, 6499-6503.

Coenye, T., Vandamme, P., 2003. Diversity and significance of Burkholderia species occupying diverse ecological niches. Environ. Microbiol. 5, 719-729.

Coenye, T., Vandamme, P., Govan, J.R., LiPuma, J.J., 2001. Taxonomy and identification of the Burkholderia cepacia complex. J. Clin. Microbiol. 39, 3427-3436.

Cooper, E.L., Kauschke, E., Cossarizza, A., 2002. Digging for innate immunity since Darwin and Metchnikoff. Bioessays 24, 319-333.

Costa, A., Jan, E., Sarnow, P., Schneider, D., 2009. The Imd pathway is involved in antiviral immune responses in Drosophila. PLoS ONE 4, e7436.

Cruz-Migoni, A., Ruzheinikov, S.N., Sedelnikova, S.E., Obeng, B., Chieng, S., Mohamed, R., Nathan, S., Baker, P.J., Rice, D.W., 2011. Cloning, purification and

crystallographic analysis of a hypothetical protein, BPSL1549, from Burkholderia pseudomallei. Acta crystallographica. Section F, Structural biology and crystallization communications 67, 1623-1626.

Currie, B., Dance, D., Cheng, A., 2008. The global distribution of Burkholderia pseudomallei and melioidosis: an update. Trans. R. Soc. Trop. Med. Hyg. 102, S1-S4.

Currie, B.J., Fisher, D.A., Howard, D.M., Burrow, J.N., Lo, D., Selva-Nayagam, S., Anstey, N.M., Huffam, S.E., Snelling, P.L., Marks, P.J., Stephens, D.P., Lum, G.D., Jacups, S.P., Krause, V.L., 2000a. Endemic melioidosis in tropical northern Australia: a 10-year prospective study and review of the literature. Clin. Infect. Dis. 31, 981-986.

Currie, B.J., Fisher, D.A., Howard, D.M., Burrow, J.N., Selvanayagam, S., Snelling, P.L., Anstey, N.M., Mayo, M.J., 2000b. The epidemiology of melioidosis in Australia and Papua New Guinea. Acta Trop. 74, 121-127.

Cuttell, L., Vaughan, A., Silva, E., Escaron, C., 2008. Undertaker, a Drosophila Junctophilin, Links Draper-Mediated Phagocytosis and Calcium Homeostasis. Cell 135, 524–534.

D'Argenio, D.A., Gallagher, L.A., Berg, C.A., Manoil, C., 2001. Drosophila as a model host for Pseudomonas aeruginosa infection. J. Bacteriol. 183, 1466-1471.

Daish, T.J., Mills, K., Kumar, S., 2004. Drosophila caspase DRONC is required for specific developmental cell death pathways and stress-induced apoptosis. Dev. Cell 7, 909-915.

Dance, D.A., Wuthiekanun, V., Chaowagul, W., White, N.J., 1989. The antimicrobial susceptibility of Pseudomonas pseudomallei. Emergence of resistance in vitro and during treatment. The Journal of antimicrobial chemotherapy 24, 295-309.

De Gregorio, E., Spellman, P.T., Tzou, P., Rubin, G.M., Lemaitre, B., 2002. The Toll and Imd pathways are the major regulators of the immune response in Drosophila. EMBO J. 21, 2568-2579.

De Verno, P., Chadwick, J., Aston, W., Dunphy, G., 1984. The generation of an antibacterial activity from the fat body and hemolymph of non-immunized larvae of. Dev. Comp. Immunol. 8, 537-546.

Defaye, A., Evans, I., Crozatier, M.l., Wood, W., Lemaitre, B., Leulier, F.o., 2009. Genetic Ablation of Drosophila Phagocytes Reveals Their Contribution to Both Development and Resistance to Bacterial Infection. Journal of Innate Immunity 1, 322-334.

Delaney, J.R., Stöven, S., Uvell, H., Anderson, K.V., Engström, Y., Mlodzik, M., 2006. Cooperative control of Drosophila immune responses by the JNK and NF-kappaB signaling pathways. EMBO J. 25, 3068-3077.

Deretic, V., Levine, B., 2009. Autophagy, immunity, and microbial adaptations. Cell host & microbe 5, 527-549.

Diamond, G., Zasloff, M., Eck, H., Brasseur, M., Maloy, W.L., Bevins, C.L., 1991. Tracheal antimicrobial peptide, a cysteine-rich peptide from mammalian tracheal mucosa: peptide isolation and cloning of a cDNA. PNAS 88, 3952-3956.

Dimarcq, J.L., Hoffmann, D., Meister, M., Bulet, P., Lanot, R., Reichhart, J.M., Hoffmann, J.A., 1994. Characterization and transcriptional profiles of a Drosophila gene encoding an insect defensin. A study in insect immunity. Eur. J. Biochem. 221, 201-209.

Dimarcq, J.L., Keppi, E., Dunbar, B., Lambert, J., Reichhart, J.M., Hoffmann, D., Rankine, S.M., Fothergill, J.E., Hoffmann, J.A., 1988. Insect immunity. Purification and characterization of a family of novel inducible antibacterial proteins from immunized larvae of the dipteran Phormia terranovae and complete amino-acid sequence of the predominant member, diptericin A. Eur. J. Biochem. 171, 17-22.

Dionne, M.S., Ghori, N., Schneider, D.S., 2003. Drosophila melanogaster is a genetically tractable model host for Mycobacterium marinum. Infect. Immun. 71, 3540-3550.

Dionne, M.S., Pham, L.N., Shirasu-Hiza, M., Schneider, D.S., 2006. Akt and foxo Dysregulation Contribute to Infection-Induced Wasting in Drosophila. Curr. Biol. 16, 1977–1985.

Dostert, C., Jouanguy, E., Irving, P., Troxler, L., Galiana-Arnoux, D., Hetru, C., Hoffmann, J.A., Imler, J.-L., 2005. The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of drosophila. Nat. Immunol. 6, 946-953.

Duffy, J., 2002. GAL4 system in Drosophila: a fly geneticist's Swiss army knife. Genesis 34, 1-15.

Dunn, P.E., Dai, W., Kanost, M.R., Geng, C.X., 1985. Soluble peptidoglycan fragments stimulate antibacterial protein synthesis by fat body from larvae of Manduca sexta. Dev. Comp. Immunol. 9, 559-568.

Ellis, R.C., Zabrowarny, L.A., 1993. Safer staining method for acid fast bacilli. J. Clin. Pathol. 46, 559-560.

Elrod-Erickson, M., Mishra, S., Schneider, D., 2000. Interactions between the cellular and humoral immune responses in Drosophila. Curr. Biol. 10, 781–784.

Evans, C.J., Hartenstein, V., Banerjee, U., 2003. Thicker than blood: conserved mechanisms in Drosophila and vertebrate hematopoiesis. Dev. Cell 5, 673-690.

Falb, D., Maniatis, T., 1992. A conserved regulatory unit implicated in tissue-specific gene expression in Drosophila and man. Genes Dev. 6, 454-465.

Fauvarque, M.O., Bergeret, E., Chabert, J., Dacheux, D., Satre, M., Attree, I., 2002. Role and activation of type III secretion system genes in Pseudomonas aeruginosa-induced Drosophila killing. Microb. Pathog. 32, 287-295.

Fehlbaum, P., Bulet, P., Michaut, L., Lagueux, M., Broekaert, W.F., Hetru, C., Hoffmann, J.A., 1994. Insect immunity. Septic injury of Drosophila induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides. J. Biol. Chem. 269, 33159-33163.

Foley, E., O'Farrell, P.H., 2004. Functional dissection of an innate immune response by a genome-wide RNAi screen. PLoS Biol. 2, E203.

Fontana, L., Partridge, L., Longo, V.D., 2010. Extending Healthy Life Span--From Yeast to Humans. Science 328, 321-326.

Franc, N.C., Dimarcq, J.L., Lagueux, M., Hoffmann, J., Ezekowitz, R.A., 1996. Croquemort, a novel Drosophila hemocyte/macrophage receptor that recognizes apoptotic cells. Immunity 4, 431-443.

Franc, N.C., Heitzler, P., Ezekowitz, R.A., White, K., 1999a. Requirement for croquemort in phagocytosis of apoptotic cells in Drosophila. Science 284, 1991-1994.

Franc, N.C., White, K., Ezekowitz, R.A.B., 1999b. Phagocytosis and development: back to the future. Curr. Opin. Immunol. 11, 47–52.

Freeman, M.R., Delrow, J., Kim, J., Johnson, E., Doe, C.Q., 2003. Unwrapping glial biology: Gcm target genes regulating glial development, diversification, and function. Neuron 38, 567-580.

Galko, M.J., Krasnow, M.A., 2004. Cellular and genetic analysis of wound healing in Drosophila larvae. PLoS Biol. 2, e239.

Gao, L., Laval, F., Lawson, E., Groger, R., Woodruff, A., Morisaki, J.H., Cox, J.S., Daffe, M., Brown, E.J., 2003. Requirement for kasB in Mycobacterium mycolic acid biosynthesis, cell wall impermeability and intracellular survival: implications for therapy. Mol. Microbiol. 49, 1547–1563.

Garver, L.S., Wu, J., Wu, L.P., 2006. The peptidoglycan recognition protein PGRP-SC1a is essential for Toll signaling and phagocytosis of Staphylococcus aureus in Drosophila. PNAS 103, 660-665.

Gaumer, S., Guénal, I., Brun, S., Théodore, L., Mignotte, B., 2000. Bcl-2 and Bax mammalian regulators of apoptosis are functional in Drosophila. Cell Death Differ. 7, 804-814.

Geisler, R., Bergmann, A., Hiromi, Y., Nüsslein-Volhard, C., 1992. cactus, a gene involved in dorsoventral pattern formation of Drosophila, is related to the I kappa B gene family of vertebrates. Cell 71, 613-621.

Gendrin, M., Welchman, D.P., Poidevin, M., Hervé, M., Lemaitre, B., 2009. Long-range activation of systemic immunity through peptidoglycan diffusion in Drosophila. PLoS Path. 5, e1000694.

Georgel, P., Naitza, S., Kappler, C., Ferrandon, D., 2001. Drosophila Immune Deficiency (IMD) Is a Death Domain Protein that Activates Antibacterial Defense and Can Promote Apoptosis. Dev. Cell 1, 503–514.

Glass, M.B., Gee, J.E., Steigerwalt, A.G., Cavuoti, D., Barton, T., Hardy, R.D., Godoy, D., Spratt, B.G., Clark, T.A., Wilkins, P.P., 2006. Pneumonia and septicemia caused by Burkholderia thailandensis in the United States. J. Clin. Microbiol. 44, 4601-4604.

Glise, B., Miller, C.A., Crozatier, M., Halbisen, M.A., Wise, S., Olson, D.J., Vincent, A., Blair, S.S., 2005. Shifted, the Drosophila ortholog of Wnt inhibitory factor-1, controls the distribution and movement of Hedgehog. Dev. Cell 8, 255-266.

Gorfinkiel, N., Sierra, J., Callejo, A., Ibañez, C., 2005. The Drosophila Ortholog of the Human Wnt Inhibitor Factor Shifted Controls the Diffusion of Lipid-Modified Hedgehog. Dev. Cell 8, 241–253.

Goto, A., Kadowaki, T., Kitagawa, Y., 2003. Drosophila hemolectin gene is expressed in embryonic and larval hemocytes and its knock down causes bleeding defects. Dev. Biol. 264, 582-591.

Goto, A., Kumagai, T., Kumagai, C., Hirose, J., Narita, H., Mori, H., Kadowaki, T., Beck, K., Kitagawa, Y., 2001. A Drosophila haemocyte-specific protein, hemolectin, similar to human von Willebrand factor. Biochem. J. 359 99-108.

Gottar, M., Gobert, V., Michel, T., Belvin, M., Duyk, G., Hoffmann, J.A., Ferrandon, D., Royet, J., 2002. The Drosophila immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein. Nature 416, 640-644.

Greenberg, S., Grinstein, S., 2002. Phagocytosis and innate immunity. Curr. Opin. Immunol. 14, 136–145.

Haase, A., Janzen, J., Barrett, S., Currie, B., 1997. Toxin production by Burkholderia pseudomallei strains and correlation with severity of melioidosis. J. Med. Microbiol. 46, 557-563.

Hagedorn, M., Soldati, T., 2007. Flotillin and RacH modulate the intracellular immunity of Dictyostelium to Mycobacterium marinum infection. Cell. Microbiol. 9, 2716-2733.

Haraga, A., West, T.E., Brittnacher, M.J., Skerrett, S.J., Miller, S.I., 2008. Burkholderia thailandensis as a model system for the study of the virulence-associated type III secretion system of Burkholderia pseudomallei. Infect. Immun. 76, 5402.

Hashimoto, C., Hudson, K.L., Anderson, K.V., 1988. The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell 52, 269-279.

Hassan, M.R.A., Pani, S.P., Peng, N.P., Voralu, K., Vijayalakshmi, N., Mehanderkar, R., Aziz, N.A., Michael, E., 2010. Incidence, risk factors and clinical epidemiology of melioidosis: a complex socio-ecological emerging infectious disease in the Alor Setar region of Kedah, Malaysia. BMC Infect. Dis. 10, 302.

Häussler, S., Nimtz, M., Domke, T., Wray, V., Steinmetz, I., 1998. Purification and characterization of a cytotoxic exolipid of Burkholderia pseudomallei. Infect. Immun. 66, 1588-1593.

Hay, B.A., Wolff, T., Rubin, G.M., 1994. Expression of baculovirus P35 prevents cell death in Drosophila. Development 120, 2121–2129.

Hedengren, M., Dushay, M.S., Ando, I., Ekengren, S., Wihlborg, M., Hultmark, D., 1999. Relish, a central factor in the control of humoral but not cellular immunity in Drosophila. Mol. Cell 4, 827-837.

Hoffmann, J.A., Reichhart, J.-M., 2002. Drosophila innate immunity: an evolutionary perspective. Nat. Immunol. 3, 121-126.

Hoffmann, W., Richter, K., 1983. A novel peptide designated PYLa and its precursor as predicted from cloned mRNA of Xenopus laevis skin. EMBO J. 2, 711–714.

Hölscher, C., Reiling, N., Schaible, U.E., 2008. Containment of aerogenic Mycobacterium tuberculosis infection in mice does not require MyD88 adaptor function for TLR2, -4 and -9. Eur. J. Immunol. 38, 680–694.

Holz, A., Bossinger, B., Strasser, T., Janning, W., Klapper, R., 2003. The two origins of hemocytes in Drosophila. Development (Cambridge, England) 130, 4955-4962.

Hrdlicka, L., Gibson, M., Kiger, A., Micchelli, C., Schober, M., Schöck, F., Perrimon, N., 2002. Analysis of twenty-four Gal4 lines in Drosophila melanogaster. Genesis 34, 51-57.

Hsieh, J.C., Kodjabachian, L., Rebbert, M.L., Rattner, A., Smallwood, P.M., Samos, C.H., Nusse, R., Dawid, I.B., Nathans, J., 1999. A new secreted protein that binds to Wnt proteins and inhibits their activities. Nature 398, 431-436.

Hultmark, D., Steiner, H., Rasmuson, T., Boman, H.G., 1980. Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of Hyalophora cecropia. Eur. J. Biochem. 106, 7-16.

Igaki, T., Kanda, H., Yamamoto-Goto, Y., Kanuka, H., 2002. Eiger, a TNF superfamily ligand that triggers the Drosophila JNK pathway. EMBO J. 21, 3009–3018.

Ip, Y., Reach, M., Engström, Y., Kadalayil, L., Tatei, K., Levine, M., 1993. Dif, a dorsal-related gene that mediates an immune response in Drosophila. Cell 75, 753-763.

Jenney, A.W., Lum, G., Fisher, D.A., Currie, B.J., 2001. Antibiotic susceptibility of Burkholderia pseudomallei from tropical northern Australia and implications for therapy of melioidosis. Int. J. Antimicrob. Agents 17, 109-113.

Jiang, C., Baehrecke, E.H., Thummel, C.S., 1997. Steroid regulated programmed cell death during Drosophila metamorphosis. Development (Cambridge, England) 124, 4673-4683.

Jones, A.L., Beveridge, T.J., Woods, D.E., 1996. Intracellular survival of Burkholderia pseudomallei. Infect. Immun. 64, 782-790.

Kaneko, T., Goldman, W.E., Mellroth, P., Steiner, H., Fukase, K., Kusumoto, S., Harley, W., Fox, A., Golenbock, D., Silverman, N., 2004. Monomeric and polymeric gram-negative peptidoglycan but not purified LPS stimulate the Drosophila IMD pathway. Immunity 20, 637-649.

Kaneko, T., Yano, T., Aggarwal, K., Lim, J.-H., Ueda, K., Oshima, Y., Peach, C., Erturk-Hasdemir, D., Goldman, W.E., Oh, B.-H., Kurata, S., Silverman, N., 2006. PGRP-LC and PGRP-LE have essential yet distinct functions in the drosophila immune response to monomeric DAP-type peptidoglycan. Nat. Immunol. 7, 715-723.

Kang, D., Liu, G., Lundström, A., Gelius, E., Steiner, H., 1998. A peptidoglycan recognition protein in innate immunity conserved from insects to humans. PNAS 95, 10078-10082.

Kespichayawattana, W., Rattanachetkul, S., Wanun, T., Utaisincharoen, P., Sirisinha, S., 2000. Burkholderia pseudomallei Induces Cell Fusion and Actin-Associated Membrane Protrusion: a Possible Mechanism for Cell-to-Cell Spreading. Infect. Immun. 68, 5377.

Kikuchi, Y., Hayatsu, M., Hosokawa, T., Nagayama, A., Tago, K., Fukatsu, T., 2012. Symbiont-mediated insecticide resistance. PNAS (acessed online ahead of print).

Kim, H.S., Schell, M.A., Yu, Y., Ulrich, R.L., Sarria, S.H., Nierman, W.C., DeShazer, D., 2005. Bacterial genome adaptation to niches: divergence of the potential virulence genes in three Burkholderia species of different survival strategies. BMC Genomics 6, 174.

Kim, Y.S., 2000. Gram-negative Bacteria-binding Protein, a Pattern Recognition Receptor for Lipopolysaccharide and beta -1,3-Glucan That Mediates the Signaling for the Induction of Innate Immune Genes in Drosophila melanogaster Cells. J. Biol. Chem. 275, 32721-32727.

Kimbrell, D.A., Beutler, B., 2001. The evolution and genetics of innate immunity. Nature reviews Genetics 2, 256-267.

Kinchen, J.M., Ravichandran, K.S., 2008. Phagosome maturation: going through the acid test. Nature Reviews Molecular Cell Biology 9, 781-795.

Kleino, A., Valanne, S., Ulvila, J., Kallio, J., Myllymäki, H., Enwald, H., Stöven, S., Poidevin, M., Ueda, R., Hultmark, D., Lemaitre, B., Rämet, M., 2005. Inhibitor of apoptosis 2 and TAK1-binding protein are components of the Drosophila Imd pathway. EMBO J. 24, 3423-3434.

Kocks, C., Cho, J., Nehme, N., Ulvila, J., Pearson, A., 2005. Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in Drosophila. Cell 123, 335–346.

Kurant, E., Axelrod, S., Leaman, D., Gaul, U., 2008. Six-microns-under acts upstream of Draper in the glial phagocytosis of apoptotic neurons. Cell 133, 498-509.

Kurucz, E., Márkus, R., Zsámboki, J., Folkl-Medzihradszky, K., Darula, Z., Vilmos, P., Udvardy, A., Krausz, I., Lukacsovich, T., Gateff, E., Zettervall, C.-J., Hultmark, D., Andó, I., 2007. Nimrod, a putative phagocytosis receptor with EGF repeats in Drosophila plasmatocytes. Curr. Biol. 17, 649-654.

Kurucz, E., Zettervall, C., Sinka, R., Vilmos, P., 2003. Hemese , a hemocyte-specific transmembrane protein, affects the cellular immune response in Drosophila. PNAS 100, 2622–2627.

Kylsten, P., Samakoviis, C., Hultmark, D., 1990. The cecropin locus in Drosophila; a compact gene cluster involved in the response to infection. EMBO J. 9, 217–224.

Lanot, R., Zachary, D., Holder, F., Meister, M., 2001. Postembryonic hematopoiesis in Drosophila. Dev. Biol. 230, 243-257.

Lazzaro, B.P., Sackton, T.B., Clark, A.G., 2006. Genetic Variation in Drosophila melanogaster Resistance to Infection: A Comparison Across Bacteria. Genetics 174, 1539-1554.

Lee, T., Luo, L., 1999. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron 22, 451-461.

Lemaitre, B., Hoffmann, J., 2007. The host defense of Drosophila melanogaster. Annu. Rev. Immunol. 25, 697-743.

Lemaitre, B., Kromer-Metzger, E., Michaut, L., Nicolas, E., Meister, M., Georgel, P., Reichhart, J.M., Hoffmann, J.A., 1995a. A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the Drosophila host defense. PNAS 92, 9465-9469.

Lemaitre, B., Meister, M., Govind, S., Georgel, P., Steward, R., Reichhart, J.M., Hoffmann, J.A., 1995b. Functional analysis and regulation of nuclear import of dorsal during the immune response in Drosophila. EMBO J. 14, 536-545.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., Hoffmann, J.A., 1996. The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 86, 973-983.

Lertpatanasuwan, N., Sermsri, K., Petkaseam, A., Trakulsomboon, S., Thamlikitkul, V., Suputtamongkol, Y., 1999. Arabinose-positive Burkholderia pseudomallei infection in humans: case report. Clin. Infect. Dis. 28, 927-928.

Leulier, F., Parquet, C., Pili-Floury, S., Ryu, J.-H., Caroff, M., Lee, W.-J., Mengin-Lecreulx, D., Lemaitre, B., 2003. The Drosophila immune system detects bacteria through specific peptidoglycan recognition. Nat. Immunol. 4, 478-484.

Levashina, E.A., Ohresser, S., Bulet, P., Reichhart, J.M., Hetru, C., Hoffmann, J.A., 1995. Metchnikowin, a novel immune-inducible proline-rich peptide from Drosophila with antibacterial and antifungal properties. Eur. J. Biochem. 233, 694-700.

Levashina, E.A., Ohresser, S., Lemaitre, B., Imler, J.L., 1998. Two distinct pathways can control expression of the gene encoding the Drosophila antimicrobial peptide metchnikowin. J. Mol. Biol. 278, 515-527.

Liang, P., Pardee, A.B., 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257, 967–971.

Limmathurotsakul, D., Wongratanacheewin, S., Teerawattanasook, N., Wongsuvan, G., Chaisuksant, S., Chetchotisakd, P., Chaowagul, W., Day, N.P.J., Peacock, S.J., 2010. Increasing Incidence of Human Melioidosis in Northeast Thailand. Am. J. Trop. Med. Hyg. 82, 1113-1117.

Manaka, J., Kuraishi, T., Shiratsuchi, A., Nakai, Y., Higashida, H., Henson, P., Nakanishi, Y., 2004. Draper-mediated and phosphatidylserine-independent phagocytosis of apoptotic cells by Drosophila hemocytes/macrophages. J. Biol. Chem. 279, 48466-48476.

Márkus, R., Laurinyecz, B., Kurucz, E., Honti, V., Bajusz, I., Sipos, B., Somogyi, K., Kronhamn, J., Hultmark, D., Ando, I., 2009. Sessile hemocytes as a hematopoietic compartment in Drosophila melanogaster. PNAS 106, 4805-4809.

Matsuyama, K., Natori, S., 1988. Molecular cloning of cDNA for sapecin and unique expression of the sapecin gene during the development of Sarcophaga peregrina. J. Biol. Chem. 263, 17117-17121.

Matthews, K., Miller, D., Kaufman, T., 1989. Developmental distribution of RNA and protein products of the α-tubulin gene family. Dev. Biol. 132, 45-61.

McClean, S., Callaghan, M., 2009. Burkholderia cepacia complex: epithelial cell-pathogen confrontations and potential for therapeutic intervention. J. Med. Microbiol. 58, 1–12.

McGurk, L., Morrison, H., Keegan, L., Sharpe, J., O'Connell, M.A., 2007. Three-Dimensional Imaging of Drosophila melanogaster. PLoS ONE 2, e834.

McMahon, A.P., Moon, R.T., 1989. Ectopic expression of the proto-oncogene int-1 in Xenopus embryos leads to duplication of the embryonic axis. Cell 58, 1075-1084.

McQuilton, P., St Pierre, S.E., Thurmond, J., FlyBase, C., 2012. FlyBase 101--the basics of navigating FlyBase. Nucleic Acids Res. 40, D706-714.

Medzhitov, R., Preston-Hurlburt, P., Janeway, C.A., 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 388, 394-397.

Michel, T., Reichhart, J.M., Hoffmann, J.A., Royet, J., 2001. Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. Nature 414, 756-759.

Minakhina, S., Steward, R., 2010. Hematopoietic stem cells in Drosophila. Development (Cambridge, England) 137, 27-31.

Moreno, E., Yan, M., Basler, K., 2002. Evolution of TNF Signaling Mechanisms: JNK-Dependent Apoptosis Triggered by Eiger, the Drosophila Homolog of the TNF Superfamily. Curr. Biol. 12, 1263–1268.

Morgan, T.H., 1910. Sex limited inheritance in Drosophila. Science 32, 120-122.

Morgan, T.H., 1934. The relation of genetics to physiology and medicine. Nobel lecture.

Morgan, T.H., Bridges, C.B., 1916. Sex-linked inheritance in Drosophila. Carnegie Institution of Washington Publication, 63-64.

Needham, A.J., Kibart, M., Crossley, H., Ingham, P.W., Foster, S.J., 2004. Drosophila melanogaster as a model host for Staphylococcus aureus infection. Microbiology 150, 2347-2355.

Nehme, N.T., Quintin, J., Cho, J.H., Lee, J., Lafarge, M.-C., Kocks, C., Ferrandon, D., 2011. Relative Roles of the Cellular and Humoral Responses in the Drosophila Host Defense against Three Gram-Positive Bacterial Infections. PLoS ONE 6, e14743.

Nelson, R.E., Fessler, L.I., Takagi, Y., Blumberg, B., Keene, D.R., Olson, P.F., Parker, C.G., Fessler, J.H., 1994. Peroxidasin: a novel enzyme-matrix protein of Drosophila development. EMBO J. 13, 3438-3447.

Nichols, Z., Vogt, R.G., 2008. The SNMP/CD36 gene family in Diptera, Hymenoptera and Coleoptera: Drosophila melanogaster, D. pseudoobscura, Anopheles gambiae, Aedes aegypti, Apis mellifera, and Tribolium castaneum. Insect Biochem. Mol. Biol. 38, 398-415.

Nicolas, E., Reichhart, J.M., Hoffmann, J.A., Lemaitre, B., 1998. In vivo regulation of the IkappaB homologue cactus during the immune response of Drosophila. The Journal of biological chemistry 273, 10463-10469.

Nierman, W.C., DeShazer, D., Kim, H.S., Tettelin, H., Nelson, K.E., Feldblyum, T., Ulrich, R.L., Ronning, C.M., Brinkac, L.M., Daugherty, S.C., Davidsen, T.D., Deboy, R.T., Dimitrov, G., Dodson, R.J., Durkin, A.S., Gwinn, M.L., Haft, D.H., Khouri, H., Kolonay, J.F., Madupu, R., Mohammoud, Y., Nelson, W.C., Radune, D., Romero, C.M., Sarria, S., Selengut, J., Shamblin, C., Sullivan, S.A., White, O., Yu, Y., Zafar, N., Zhou, L., Fraser, C.M., 2004. Structural flexibility in the Burkholderia mallei genome. Proc. Natl. Acad. Sci. U. S. A. 101, 14246-14251.

O'Donnell, K.H., Chen, C.T., Wensink, P.C., 1994. Insulating DNA directs ubiquitous transcription of the Drosophila melanogaster alpha 1-tubulin gene. Mol. Cell. Biol 14, 6398-6408.

Okada, M., Natori, S., 1985. Primary structure of sarcotoxin I, an antibacterial protein induced in the hemolymph of Sarcophaga peregrina (flesh fly) larvae. J. Biol. Chem. 260, 7174-7177.

Pavlidis, P., Ramaswami, M., Tanouye, M.A., 1994. The Drosophila easily shocked gene: a mutation in a phospholipid synthetic pathway causes seizure, neuronal failure, and paralysis. Cell 79, 23-33.

Pearson, A., Lux, A., 1995. Expression cloning of dSR-CI, a class C macrophage-specific scavenger receptor from Drosophila melanogaster. PNAS 92, 4056-4060.

Philips, J.A., Rubin, E.J., Perrimon, N., 2005. Drosophila RNAi screen reveals CD36 family member required for mycobacterial infection. Science 309, 1251-1253.

Pilatz, S., Breitbach, K., Hein, N., Fehlhaber, B., Schulze, J., Brenneke, B., Eberl, L., Steinmetz, I., 2006. Identification of Burkholderia pseudomallei genes required for the intracellular life cycle and in vivo virulence. Infect. Immun. 74, 3576-3586.

Pimenta, A.d.L., Martino, P.D., Bouder, E.L., Hulen, C., Blight, M.A., 2003. In vitro identification of two adherence factors required for in vivo virulence of Pseudomonas fluorescens. Microb. Infect. 5, 1177-1187.

Pozos, T.C., Ramakrishnan, L., Ramakrishan, L., 2004. New models for the study of Mycobacterium-host interactions. Curr. Opin. Immunol. 16, 499-505.

Pye, A.E., Boman, H.G., 1977. Insect immunity. III. Purification and partial characterization of immune protein P5 from hemolymph of Hyalophora cecropia pupae. Infect. Immun. 17, 408-414.

Radaeva, T.V., Nikonenko, B.V., Mischenko, V.V., Averbakh, M.M., Apt, A.S., 2005. Direct comparison of low-dose and Cornell-like models of chronic and reactivation tuberculosis in genetically susceptible I/St and resistant B6 mice. Tuberculosis 85, 65-72.

Ramakrishnan, L., Valdivia, R.H., McKerrow, J.H., Falkow, S., 1997. Mycobacterium marinum causes both long-term subclinical infection and acute disease in the leopard frog (Rana pipiens). Infect. Immun. 65, 767-773.

Rämet, M., Manfruelli, P., Pearson, A., Mathey-Prevot, B., Ezekowitz, R.A., 2002. Functional genomic analysis of phagocytosis and identification of a Drosophila receptor for *E. coli*. Nature 416, 644-648.

Rämet, M., Pearson, A., Manfruelli, P., Li, X., Koziel, H., Göbel, V., Chung, E., Krieger, M., Ezekowitz, R.A., 2001. Drosophila scavenger receptor CI is a pattern recognition receptor for bacteria. Immunity 15, 1027-1038.

Ranz, J.M., Casals, F., Ruiz, A., 2001. How malleable is the eukaryotic genome? Extreme rate of chromosomal rearrangement in the genus Drosophila. Genome Res. 11, 230-239.

Rizki, T., Rizki, R.M., 1980. Properties of the larval hemocytes of Drosophila melanogaster. Experientia 36, 1223-1226.

Rogers, S.L., Rogers, G.C., 2008. Culture of Drosophila S2 cells and their use for RNAi-mediated loss-of-function studies and immunofluorescence microscopy. Nature Protocols 3, 606-611.

Rotz, L.D., Khan, A.S., Lillibridge, S.R., Ostroff, S.M., Hughes, J.M., 2002. Public health assessment of potential biological terrorism agents. Emerging Infect. Dis. 8, 225-230.

Rutschmann, S., Jung, A.C., Zhou, R., Silverman, N., Hoffmann, J.A., Ferrandon, D., 2000. Role of Drosophila IKK gamma in a toll-independent antibacterial immune response. Nat. Immunol. 1, 342-347.

Samakovlis, C., Kimbrell, D.A., Kylsten, P., Engström, A., Hultmark, D., 1990. The immune response in Drosophila: pattern of cecropin expression and biological activity. EMBO J. 9, 2969-2976.

Sarkar-Tyson, M., Smither, S.J., Harding, S.V., Atkins, T.P., Titball, R.W., 2009. Protective efficacy of heat-inactivated B. thailandensis, B. mallei or B. pseudomallei against experimental melioidosis and glanders. Vaccine 27, 4447-4451.

Sass, A., Marchbank, A., Tullis, E., Lipuma, J.J., Mahenthiralingam, E., 2011. Spontaneous and evolutionary changes in the antibiotic resistance of Burkholderia cenocepacia observed by global gene expression analysis. BMC Genomics 12, 373.

Savill, J., Hogg, N., Ren, Y., Haslett, C., 1992. Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. J. Clin. Invest. 90, 1513-1522.

Schaible, U.E., Winau, F., Sieling, P.A., Fischer, K., Collins, H.L., Hagens, K., Modlin, R.L., Brinkmann, V., Kaufmann, S.H.E., 2003. Apoptosis facilitates antigen

presentation to T lymphocytes through MHC-I and CD1 in tuberculosis. Nat. Med. 9, 1039-1046.

Schneider, D., Shahabuddin, M., 2000. Malaria parasite development in a Drosophila model. Science 288, 2376-2379.

Schneider, D.S., Ayres, J.S., Brandt, S.M., Costa, A., Dionne, M.S., Gordon, M.D., Mabery, E.M., Moule, M.G., Pham, L.N., Shirasu-Hiza, M.M., 2007. Drosophila eiger mutants are sensitive to extracellular pathogens. PLoS Path. 3, e41.

Schneider, D.S., Hudson, K.L., Lin, T.Y., Anderson, K.V., 1991. Dominant and recessive mutations define functional domains of Toll, a transmembrane protein required for dorsal-ventral polarity in the Drosophila embryo. Genes Dev. 5, 797-807.

Schneider, I., 1972. Cell lines derived from late embryonic stages of Drosophila melanogaster. J. Embryol. Exp. Morphol. 27, 353-365.

Schnetzer, J.W., Tyler, M.S., 1996. Endogenous beta-galactosidase activity in the larval, pupal, and adult stages of the fruit fly, Drosophila melanogaster, indicates need for caution in lacZ fusion-gene studies. Biol. Bull. 190, 173-187.

Schultz, M.G., 2011. Robert Koch. Emerging Infect. Dis. 17, 547-549.

Schulz, C., Gomez Perdiguero, E., Chorro, L., Szabo-Rogers, H., Cagnard, N., Kierdorf, K., Prinz, M., Wu, B., Jacobsen, S.E.W., Pollard, J.W., Frampton, J., Liu, K.J., Geissmann, F., 2012. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. Science (New York, NY) 336, 86-90.

Schwarz, S., West, T.E., Boyer, F., Chiang, W.C., Carl, M.A., Hood, R.D., Rohmer, L., Tolker-Nielsen, T., Skerrett, S.J., Mougous, J.D., 2010. Burkholderia type VI secretion systems have distinct roles in eukaryotic and bacterial cell interactions. PLoS Path. 6, e1001068.

Schweizer, H.P., Peacock, S.J., 2008. Antimicrobial Drug–Selection Markers for Burkholderia pseudomallei and B. mallei. Emerging Infect. Dis. 14, 1689-1692.

Seroude, L., Brummel, T., Kapahi, P., Benzer, S., 2002. Spatio-temporal analysis of gene expression during aging in Drosophila melanogaster. Aging Cell 1, 47-56.

Shelly, S., Lukinova, N., Bambina, S., Berman, A., Cherry, S., 2009. Autophagy is an essential component of Drosophila immunity against vesicular stomatitis virus. Immunity 30, 588-598.

Shields, G., Sang, J.H., 1970. Characteristics of five cell types appearing during in vitro culture of embryonic material from Drosophila melanogaster. J. Embryol. Exp. Morphol. 23, 53-69.

Silverman, N., Zhou, R., Erlich, R.L., Hunter, M., Bernstein, E., Schneider, D., Maniatis, T., 2003. Immune activation of NF-kappaB and JNK requires Drosophila TAK1. J. Biol. Chem. 278, 48928-48934.

Silverstein, R.L., Febbraio, M., 2009. CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior. Science signaling 2, re3.

Simon, A., Kullberg, B.J., Tripet, B., Boerman, O.C., Zeeuwen, P., van der Ven-Jongekrijg, J., Verweij, P., Schalkwijk, J., Hodges, R., van der Meer, J.W.M., Netea, M.G., 2008. Drosomycin-like defensin, a human homologue of Drosophila melanogaster drosomycin with antifungal activity. Antimicrob. Agents Chemother. 52, 1407-1412.

Sluss, H.K., Han, Z., Barrett, T., Goberdhan, D.C., Wilson, C., Davis, R.J., Ip, Y.T., 1996. A JNK signal transduction pathway that mediates morphogenesis and an immune response in Drosophila. Genes Dev. 10, 2745-2758.

Smith, M.D., Wuthiekanun, V., Walsh, A.L., White, N.J., 1995. Quantitative recovery of Burkholderia pseudomallei from soil in Thailand. Trans. R. Soc. Trop. Med. Hyg. 89, 488-490.

Sofer, W., 1987. Analysis of alcohol dehydrogenase gene expression in Drosophila. Annu. Rev. Genet. 21, 203-225.

Steiner, H., Hultmark, D., Engström, A., Bennich, H., Boman, H.G., 1981. Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature 292, 246-248.

Stevens, J.M., Ulrich, R.L., Taylor, L.A., Wood, M.W., DeShazer, D., Stevens, M.P., Galyov, E.E., 2005a. Actin-binding proteins from Burkholderia mallei and Burkholderia thailandensis can functionally compensate for the actin-based motility defect of a Burkholderia pseudomallei bimA mutant. J. Bacteriol. 187, 7857-7862.

Stevens, M.P., Friebel, A., Taylor, L.A., Wood, M.W., Brown, P.J., Hardt, W.-D., Galyov, E.E., 2003. A Burkholderia pseudomallei type III secreted protein, BopE, facilitates bacterial invasion of epithelial cells and exhibits guanine nucleotide exchange factor activity. J. Bacteriol. 185, 4992-4996.

Stevens, M.P., Haque, A., Atkins, T., Hill, J., Wood, M.W., Easton, A., Nelson, M., Underwood-Fowler, C., Titball, R.W., Bancroft, G.J., Galyov, E.E., 2004. Attenuated virulence and protective efficacy of a Burkholderia pseudomallei bsa type III secretion mutant in murine models of melioidosis. Microbiology 150, 2669-2676.

Stevens, M.P., Stevens, J.M., Jeng, R.L., Taylor, L.A., Wood, M.W., Hawes, P., Monaghan, P., Welch, M.D., Galyov, E.E., 2005b. Identification of a bacterial factor required for actin-based motility of Burkholderia pseudomallei. Mol. Microbiol. 56, 40-53.

Stofanko, M., Kwon, S.Y., Badenhorst, P., 2008. A misexpression screen to identify regulators of Drosophila larval hemocyte development. Genetics 180, 253-267.

Stramer, B., Wood, W., Galko, M., Redd, M., 2005. Live imaging of wound inflammation in Drosophila embryos reveals key roles for small GTPases during in vivo cell migration. J. Cell Biol. 168, 567–573.

Stronach, B., 2005. Dissecting JNK signaling, one KKKinase at a time. Dev. Dyn. 232, 575–584.

Stuart, L.M., Deng, J., Silver, J.M., Takahashi, K., Tseng, A.A., Hennessy, E.J., Ezekowitz, R.A.B., Moore, K.J., 2005. Response to Staphylococcus aureus requires

CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain. The Journal of cell biology 170, 477-485.

Stuart, L.M., Ezekowitz, R.A.B., 2005. Phagocytosis: elegant complexity. Immunity 22, 539-550.

Sturtevant, A.H., 1913. The linear arrangement of six sex-linked factors in Drosophila, as shown by their mode of association. J. Exp. Zool. 14, 43-59.

Suputtamongkol, Y., Chaowagul, W., Chetchotisakd, P., Lertpatanasuwun, N., Intaranongpai, S., Ruchutrakool, T., Budhsarawong, D., Mootsikapun, P., Wuthiekanun, V., Teerawatasook, N., Lulitanond, A., 1999. Risk factors for melioidosis and bacteremic melioidosis. Clin. Infect. Dis. 29, 408-413.

Swaim, L.E., Connolly, L.E., Volkman, H.E., Humbert, O., Born, D.E., Ramakrishnan, L., 2006. Mycobacterium marinum infection of adult zebrafish causes caseating granulomatous tuberculosis and is moderated by adaptive immunity. Infect. Immun. 74, 6108-6117.

Swarup, S., Verheyen, E.M., 2012. Wnt/Wingless Signaling in Drosophila. Cold Spring Harbor perspectives in biology.

Tacke, F., Randolph, G.J., 2006. Migratory fate and differentiation of blood monocyte subsets. Immunobiology 211, 609-618.

Tepass, U., Fessler, L.I., Aziz, A., Hartenstein, V., 1994. Embryonic origin of hemocytes and their relationship to cell death in Drosophila. Development (Cambridge, England) 120, 1829-1837.

Tønjum, T., Welty, D.B., Jantzen, E., Small, P.L., 1998. Differentiation of Mycobacterium ulcerans, *M. marinum*, and M. haemophilum: mapping of their relationships to M. tuberculosis by fatty acid profile analysis, DNA-DNA hybridization, and 16S rRNA gene sequence analysis. J. Clin. Microbiol. 36, 918-925.

Trump, B.F., Berezesky, I.K., Chang, S.H., Phelps, P.C., 1997. The pathways of cell death: oncosis, apoptosis, and necrosis. Toxicol. Pathol. 25, 82-88.

Tryselius, Y., Samakovlis, C., Kimbrell, D.A., Hultmark, D., 1992. CecC, a cecropin gene expressed during metamorphosis in Drosophila pupae. Eur. J. Biochem. 204, 395-399.

Tzou, P., Ohresser, S., Ferrandon, D., Capovilla, M., Reichhart, J.M., Lemaitre, B., Hoffmann, J.A., Imler, J.L., 2000. Tissue-specific inducible expression of antimicrobial peptide genes in Drosophila surface epithelia. Immunity 13, 737-748.

van der Wel, N., Hava, D., Houben, D., Fluitsma, D., van Zon, M., Pierson, J., Brenner, M., Peters, P., 2007. M. tuberculosis and M. leprae Translocate from the Phagolysosome to the Cytosol in Myeloid Cells. Cell 129, 1287–1298.

Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G.F., Chater, K.F., van Sinderen, D., 2007. Genomics of Actinobacteria: Tracing the Evolutionary History of an Ancient Phylum. Microbiol. Mol. Biol. Rev. 71, 495-548.

Vergne, I., Chua, J., Singh, S.B., Deretic, V., 2004. Cell biology of Mycobacterium tuberculosis phagosome. Annu. Rev. Cell. Dev. Biol. 20, 367-394.

Vorachit, M., Lam, K., Jayanetra, P., Costerton, J.W., 1993. Resistance of Pseudomonas pseudomallei growing as a biofilm on silastic discs to ceftazidime and co-trimoxazole. Antimicrob. Agents Chemother. 37, 2000-2002.

Watson, F.L., Püttmann-Holgado, R., Thomas, F., Lamar, D.L., Hughes, M., Kondo, M., Rebel, V.I., Schmucker, D., 2005. Extensive diversity of Ig-superfamily proteins in the immune system of insects. Science 309, 1874–1878.

Werner, T., Borge-Renberg, K., Mellroth, P., Steiner, H., Hultmark, D., 2003. Functional diversity of the Drosophila PGRP-LC gene cluster in the response to lipopolysaccharide and peptidoglycan. J. Biol. Chem. 278, 26319-26322.

Werner, T., Liu, G., Kang, D., Ekengren, S., Steiner, H., Hultmark, D., 2000. A family of peptidoglycan recognition proteins in the fruit fly Drosophila melanogaster. PNAS 97, 13772-13777.

White, K., Grether, M.E., Abrams, J.M., Young, L., Farrell, K., Steller, H., 1994. Genetic control of programmed cell death in Drosophila. Science (New York, NY) 264, 677-683.

White, N.J., 2003. Melioidosis. Lancet 361, 1715-1722.

Whitlock, G.C., Mark Estes, D., Torres, A.G., 2007. Glanders: off to the races with Burkholderia mallei. FEMS Microbiol. Lett. 277, 115-122.

Wicker, C., Reichhart, J.M., Hoffmann, D., Hultmark, D., Samakovlis, C., Hoffmann, J.A., 1990. Insect immunity. Characterization of a Drosophila cDNA encoding a novel member of the diptericin family of immune peptides. J. Biol. Chem. 265, 22493-22498.

Wiersinga, W.J., de Vos, A.F., de Beer, R., Wieland, C.W., Roelofs, J.J.T.H., Woods, D.E., van der Poll, T., 2008. Inflammation patterns induced by different Burkholderia species in mice. Cell. Microbiol. 10, 81-87.

Wiklund, M.-L., Steinert, S., Junell, A., Hultmark, D., Stöven, S., 2009. The N-terminal half of the Drosophila Rel/NF-kappaB factor Relish, REL-68, constitutively activates transcription of specific Relish target genes. Dev. Comp. Immunol. 33, 690-696.

Wood, W., Jacinto, A., 2007. Drosophila melanogaster embryonic haemocytes: masters of multitasking. Nature Reviews Molecular Cell Biology 8, 542-551.

Yanagawa, S., Lee, J.S., 1998. Identification and Characterization of a Novel Line ofDrosophila Schneider S2 Cells That Respond to Wingless Signaling. J. Biol. Chem. 273, 32353–32359.

Yano, T., Mita, S., Ohmori, H., Oshima, Y., Fujimoto, Y., Ueda, R., Takada, H., Goldman, W.E., Fukase, K., Silverman, N., Yoshimori, T., Kurata, S., 2008. Autophagic control of listeria through intracellular innate immune recognition in drosophila. Nat. Immunol. 9, 908-916.

Yu, Y., Kim, H.S., Chua, H.H., Lin, C.H., Sim, S.H., Lin, D., Derr, A., Engels, R., DeShazer, D., Birren, B., Nierman, W.C., Tan, P., 2006. Genomic patterns of pathogen evolution revealed by comparison of Burkholderia pseudomallei, the causative agent of melioidosis, to avirulent Burkholderia thailandensis. BMC Microbiol. 6, 46.

Zasloff, M., 1987. Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. PNAS 84, 5449-5453.

Zasloff, M., 2002. Antimicrobial peptides of multicellular organisms. Nature 415, 389-395.

Zettervall, C.-J., Anderl, I., Williams, M.J., Palmer, R., Kurucz, E., Andó, I., Hultmark, D., 2004. A directed screen for genes involved in Drosophila blood cell activation. PNAS 101, 14192-14197.

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APPENDIX

Pilátová M, Dionne MS (2012) *Burkholderia thailandensis* Is Virulent in *Drosophila melanogaster*. PLoS ONE 7(11): e49745.doi:10.1371/journal.pone.0049745

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Burkholderia thailandensis Is Virulent in *Drosophila melanogaster*

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Abstract

Melioidosis is a serious infectious disease endemic to Southeast Asia and Northern Australia. This disease is caused by the Gram-negative bacterium *Burkholderia pseudomallei; Burkholderia thailandensis* is a closely-related organism known to be avirulent in humans. *B. thailandensis* has not previously been used to infect *Drosophila melanogaster*. We examined the effect of *B. thailandensis* infection on fly survival, on antimicrobial peptide expression, and on phagocytic cells. In the fruit fly, which possesses only an innate immune system, *B. thailandensis* is highly virulent, causing rapid death when injected or fed. One intriguing aspect of this infection is its temperature dependence: infected flies maintained at 25°C exhibit rapid bacterial proliferation and death in a few days, while infected animals maintained at 18°C exhibit very slow bacterial proliferation and take weeks to die; this effect is due in part to differences in immune activity of the host. Death in this infection is likely due at least in part to a secreted toxin, as injection of flies with sterile *B. thailandensis*-conditioned medium is able to kill. *B. thailandensis* infection strongly induces the expression of antimicrobial peptides, but this is insufficient to inhibit bacterial proliferation in infected flies. Finally, the function of fly phagocytes is not affected by *B. thailandensis* infection. The high virulence of *B. thailandensis* in the fly suggests the possibility that this organism is a natural pathogen of one or more invertebrates.

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Introduction

Melioidosis is a serious human and animal disease caused by the Gram-negative bacterium *Burkholderia pseudomallei*. Moist soils of rice paddies or surface water harbour this pathogen in endemic areas of Southeast Asia and Northern Australia [1–3]. Melioidosis can be contracted through damaged skin from *B. pseudomallei*-infected soil and water or by inhaling aerosolised bacteria [4]. In humans, melioidosis can manifest itself as a fever, mild or severe septicaemic pneumonia, skin and internal organ abscesses, and neurological conditions, such as brainstem encephalitis [3,5]. The treatment of melioidosis is long and frequently unsuccessful; in many cases the disease recurs [6]. Currie and colleagues conducted a 10-year study of melioidosis patients and found that approximately 86% of patients who suffer septic shock as a result of this infection die [7]. The outcome of melioidosis also depends on individual circumstances and risk factors; diabetes, chronic renal disease or alcoholism have been reported to increase the rate of death in melioidosis patients [5,8].

B. pseudomallei infection has been studied in Syrian golden hamsters to model melioidosis; in mice to understand various aspects of the bacterial pathogenicity, such as the effect of wild-type (WT) or mutant strains of *B. pseudomallei* on the survival of WT mice, and *in vitro* to gain insight into the intracellular life cycle of *B. pseudomallei* and its motility [9–11]. As this highly pathogenic bacterium is a Class B infectious agent, its study requires BSL-3 containment conditions [12]. In addition, *B. pseudomallei* is resistant to many antibiotics; restrictions on the use of antibiotics in the study of this pathogen apply [13,14]. Due to these limitations, a safer and cheaper model for the study of some aspects of melioidosis could prove invaluable.

B. pseudomallei is closely related to the non-pathogenic *Burkholderia thailandensis* [15–17]. When discovered, *B. thailandensis* was thought to be an isolate of *B. pseudomallei*; later Brett and colleagues renamed it from *B. pseudomallei*-like to its current name [15]. Although *B. thailandensis* is mostly avirulent in mammals, high doses of *B. thailandensis* E264 kill mice [18,19]. *B. thailandensis* and *B. pseudomallei* are motile, and live in soil and surface water, and are therefore adapted to similar environmental conditions [11,20,21]. Although *B. thailandensis* is not virulent in the Syrian golden hamster model [9], occasional *B. thailandensis* infections have been reported in people; in 1999 a motorcycle accident in Thailand led to melioidosis-like symptoms (here *B. thailandensis* is referred to as Ara+ *B. pseudomallei)* [22]; in the U.S., Glass and colleagues reported that *B. thailandensis* strain ATCC 700388 infection led to pneumonia and septicaemia in a 2-year old boy involved in a car accident [23].

Drosophila melanogaster (*D. melanogaster*) is a proven model for the study of various infections, such as *Mycobacterium marinum* [24], *Salmonella typhimurium* [25], and *Staphylococcus aureus* [26]. Despite the fact that no adaptive immunity has been discovered in *D.*

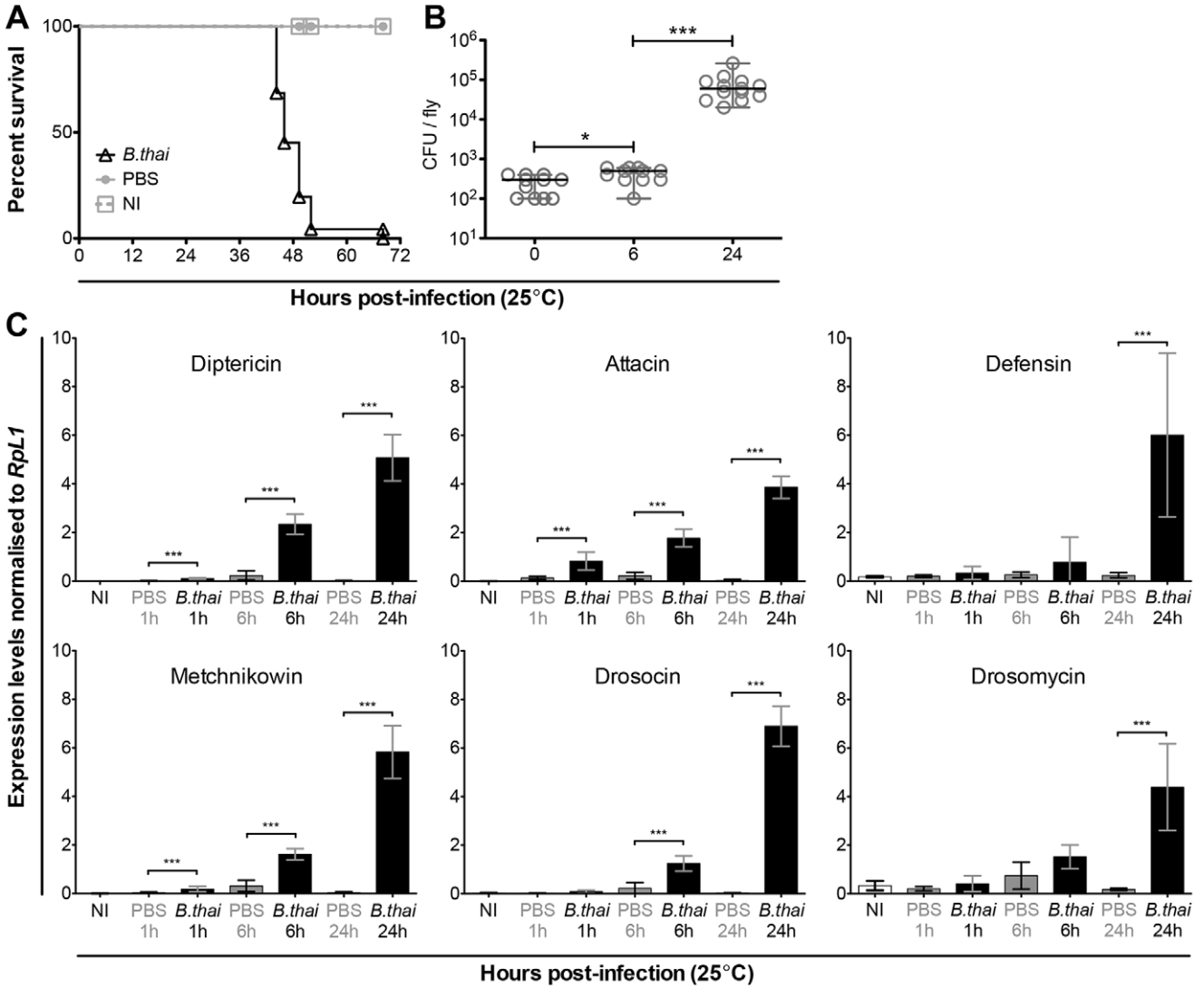

Figure 1. *B. thailandensis* infection kills WT male *D. melanogaster*, survives and grows in the host. (A) *Oregon-R* males were infected with WT *B. thailandensis* (*B. thai*) and died within 3.5 days of infection. Survival data was pooled from 3 independent experiments (n = min. 51 males per condition). Bacteria were injected at OD600 = 0.01, (approximately 250 CFU per fly). Mock-infected (PBS) controls were alive for the duration of this experiment. (B) *B. thailandensis* survived and multiplied inside infected flies. The data is based on 2 independent experiments (n = min. 11 males per time point). *B. thailandensis* was injected at a dose of OD600 = 0.01. Samples were collected at 0, 6 and 24 h p.i. and bacterial growth determined by plating dilutions of homogenised samples. Colonies were counted 24 h after the homogenate was plated and incubated at 37°C. Statistical significance of bacterial growth between time points was determined using Mann-Whitney test; * $p<0.02$ and *** $p<0.0001$. (C) *B. thailandensis* infection induced AMP expression in *D. melanogaster*. Three infection time points were analysed: 1, 6, and 24 h; controls were either mock-infected (PBS) or uninjected (NI). All tested AMPs were without exception significantly induced 24 h after infection. Levels of AMP mRNA were determined by qPCR. Statistical significance between levels of AMP expression was determined using Mann-Whitney test (GraphPad Prism); *** $p<0.001$. Data is based on 1 experiment, n = 7 males per condition; error bars represent SD.
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melanogaster, the fly is an attractive potential model host to examine the role of innate immunity in melioidosis. The interactions of *Drosophila* with the *Burkholderia cepacia* complex have also been previously examined [27,28]. However, to our knowledge, non-cepacia *Burkholderiaceae* have not previously been examined in *Drosophila*, despite the appeal of this organism as a potential model host to examine the role of innate immunity in melioidosis.

The aim of this study was to evaluate *D. melanogaster* as a model organism for the study of host-pathogen interactions and the role of the innate immune response in melioidosis. The results show that *B. thailandensis* infection in *D. melanogaster* to some extent parallels *B. pseudomallei* infection in mammalian hosts. This model thus may advance our understanding of the host-pathogen interaction in terms of innate immunity.

Materials and Methods

Fly stocks

To examine the effect of *B. thailandensis* infection on *Drosophila* survival, we used wild-type (WT) fly strains *Oregon-R* and w^{1118} (DrosDel isogenic background), and a Toll and Imd pathway simultaneous loss-of-function mutant (*Dif; Rel*). Fruit flies expressing eGFP under the control of a haemocyte-specific promoter, *hemolectin* (*HmlΔGAL4, UAS-2xeGFP*), were used as a control for

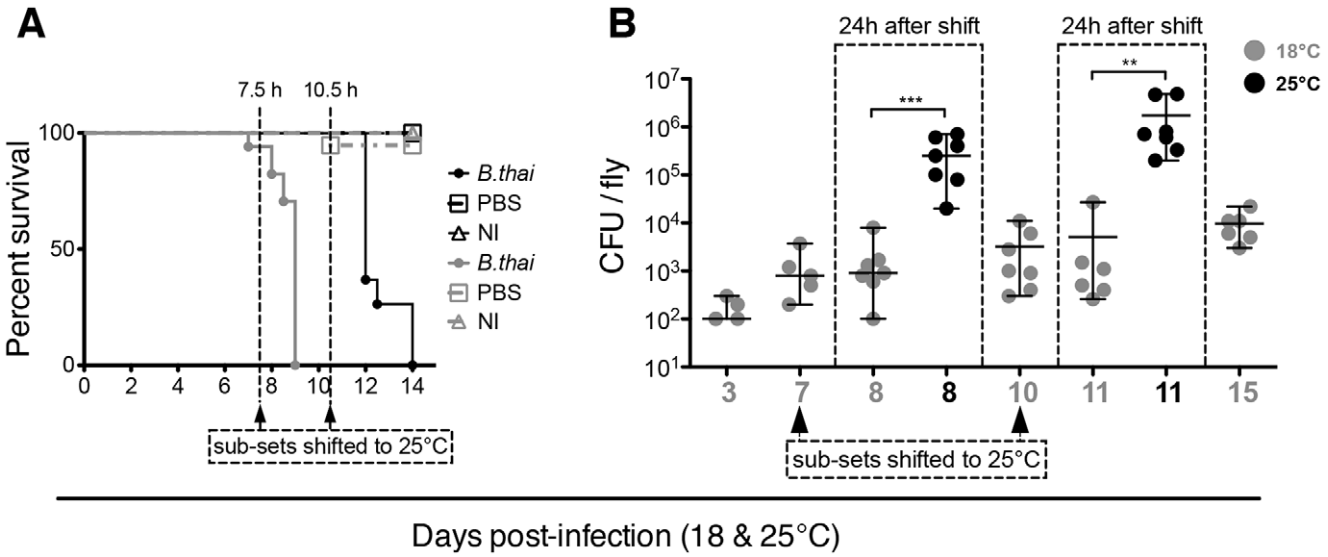

Figure 2. *B. thailandensis* growth at 18°C is slower than at 25°C. (A) Survival of wild-type flies infected with wild-type *B. thailandensis* E264 and kept at 18°C. (B) Infected and control flies were kept at 18°C, and subgroups were shifted to 25°C at time points 7.5 and 10.5 days after infection. Dead flies were counted twice a day. The result indicates that bacteria recovered at 25°C, and killed the flies fast. (C) *B. thailandensis* was injected at an initial dose of OD600 = 0.01. Flies were kept at 18°C (grey) and shifted to 25°C (black) at time points 7 and 10 days p.i. Subsets of equally treated flies were kept at 18°C as controls (grey). Samples were homogenised 24 h after shifting from 18 to 25°C to determine the growth of bacteria inside the flies. Samples were analysed at time points 3, 7, 8, 10, 11, and 15 days p.i. Bacterial growth was determined by plating dilutions of homogenised infected and control flies in PBS. Plated bacteria were left at 37°C for 24 h, when bacterial colonies were counted. Data is based on one experiments; n = 7 flies. Statistical significance of bacterial growth was determined using Mann-Whitney test (GraphPad Prism); ** $p<0.002$, *** $p<0.001$. Y-axis = log10.

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imaging experiments to show the pattern of haemocyte distribution in the dorsal side of untreated flies (NI). All infection experiments were performed in male flies because females exhibit higher levels of nonspecific mortality due to food liquefaction.

Bacterial cultures

Cultures of WT *B. thailandensis* E264 (kind gift of Madeleine Moule and Brendan Wren), WT GFP-labelled and T6SS mutant *B. thailandensis* (kind gift from the Mougous lab) [29], T3SS mutant *B. thailandensis* (AH174, AH183 and the complemented strain AH186, kind gifts from the Miller lab) [18] and *Escherichia coli* DH5α were set up from frozen stocks and cultured in standard lysogeny broth (LB) at 37°C overnight with agitation. For those survival experiments indicated in the text, WT and mutant {AH174 and ΔT6SS-(1–6)} *B. thailandensis* cultures were used at an exponential-growth phase; overnight culture was diluted 1 in 10 in fresh LB and incubated for three hours at 37°C with shaking. For infection assays with phosphate buffered saline (PBS) as a control, bacterial cultures were harvested by centrifugation at 2400×g for 4 minutes at room temperature, re-suspended in PBS and calibrated using a spectrophotometer (Eppendorf); for infections with LB as a control, cultures were kept in the original growth medium and calibrated with LB to the desired density. *B. thailandensis* was calibrated to OD_{600} of 0.01, which represents approximately 250 CFU per fly when injected. *E. coli* was calibrated to OD_{600} of 1. To ensure that the LB broth was not contaminated, separate bacteria-free LB was prepared and treated in exactly the same way as LB-containing bacteria.

Heat-inactivated *B. thailandensis* stock was prepared as per Sarkar-Tyson et al. [30]. The protocol was slightly modified; inactivated cultures were kept as frozen stocks at −80°C. Heat-killed *B. thailandensis* was tested for viability by incubating in liquid LB at 37°C for 48 hours (h) with shaking.

For experiments with *B. thailandensis*-conditioned medium (CM), overnight cultures were harvested by centrifugation, but in this case the supernatant was removed into a new tube and sterile-filtered using a 0.2 μm filter (Sartorius). To ensure that the CM contained no live bacteria, a portion of the same CM that had been injected into flies was plated on standard LB agar plate and kept at 37°C for 48 hours. As a control, 5 ml of LB was treated and processed in precisely the same way as the CM, and used for mock-infections as well as for plating.

For additional experiments, CM was heat-treated (H/T) as per modified Sarkar-Tyson et al. [30] protocol. To ensure that the H/T CM contained no live bacteria, a portion of the same H/T CM that had been injected into flies was inoculated into LB and incubated at 37°C for 24 hours. As a control, H/T LB was treated and processed in precisely the same way as the H/T CM, and used for mock-infections.

Survival assays

Flies were kept in 30 ml tubes with roughly 8 ml *Drosophila* medium (10% brewer's yeast, 8% fructose, 2% polenta, 0.8% agar, supplemented with nipagin and propionic acid). Eclosed males of the required genotypes were collected from these tubes once a day and transferred into tubes containing fresh food. They were allowed to mature for 5–10 days prior to injection. Mature male flies were injected with a calibrated suspension of overnight bacterial culture or sterile filtered *B. thailandensis*-CM or H/T CM. Mock-infected control flies were injected with PBS, LB or H/T LB, and all injections were done using a Picospritzer® III microinjector (Intracel). In most experiments, a third set of uninjected males was kept as an untreated control. Depending on the experiment, the infected and control flies were kept at 18 or 25°C; dead flies were counted at least twice a day.

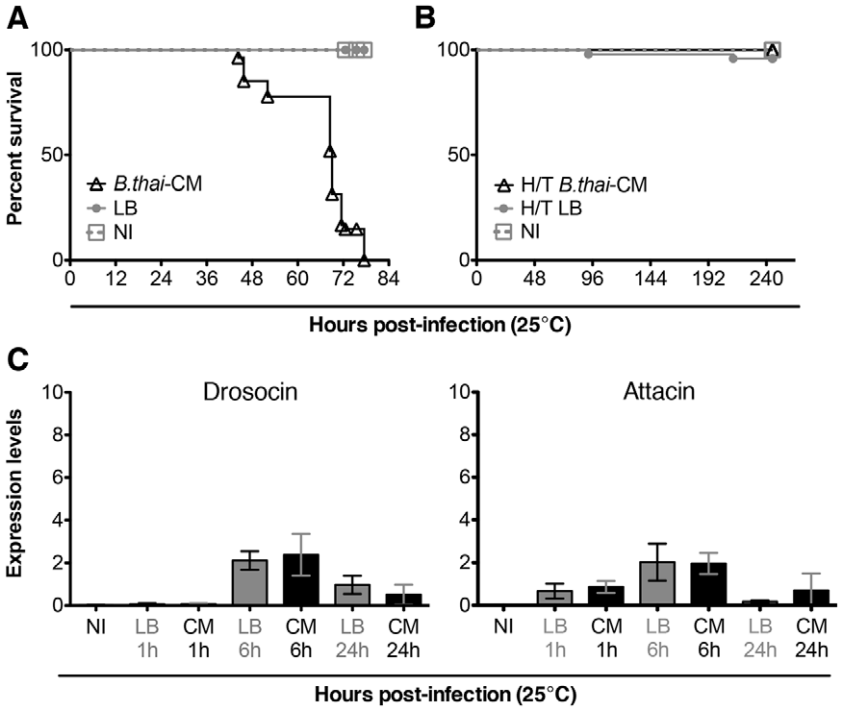

Figure 3. Sterile *B. thailandensis*-conditioned medium kills flies. (A) *Oregon-R* males injected with sterile-filtered *B. thailandensis*-conditioned medium (CM) died after injection. Mock-infected (LB) and uninjected (NI) controls continued to live at least for the duration of this experiment; data is based on 3 independent experiments, n = min. 56 males per condition. (B) Heat-treated conditioned medium did not kill flies; data is based on 2 independent experiments, n = min. 49 males per condition. (C) Antimicrobial peptides, Drosocin and Attacin, were not induced by *B. thailandensis*-conditioned medium. The levels of AMP mRNA were determined by qPCR; data is based on 1 experiment, n = 7 males per condition; error bars represent SD.
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Bacterial burden

Flies were infected as per survival assays. Infected flies were homogenised in PBS at 0, 6 and 24 h post-infection (p.i.); 0 h p.i. was the 'input control'. One tenth of the homogenate was diluted, 1:10, 1:100, 1:1000, 1:10000, and plated on sterile LB agar plates. The plates were kept at 37°C and colonies counted 24 h after plating. Statistical significance of bacterial growth between time points was determined using Mann-Whitney test (GraphPad Prism).

Feeding assays

Flies were maintained and selected as per survival assays. Overnight culture of *B. thailandensis* was spun at 4°C at maximum speed for 5 minutes to obtain a bacterial pellet. The spent medium was removed and the bacteria were resuspended in 1/50x PBS supplemented with 1 mM each $CaCl_2$ and $MgCl_2$. Fly food was prepared using dry mix containing 8.5 g fructose (Fruisana), 6.1 g dry milk powder (Marvel), 18 g Smash brand dehydrated mashed potatoes. 1 g of this dry mix was placed into each fly vial and 2 ml of bacterial suspension was added. Control food was prepared using the dry mix and PBS. The fly food was ready to use in less than 30 minutes. Experimental and control flies were put on the appropriate food and counted daily.

mRNA extraction and cDNA synthesis

Total mRNA was extracted from infected and control flies using 100 µl of Trizol reagent (Invitrogen) as per the manufacturer's protocol. Complementary DNA (cDNA) was synthesized using the First Strand cDNA Synthesis Kit (Fermentas). The kit was used according to the manufacturer's instructions. Random Hexamers were contained in the First Strand cDNA Synthesis Kit, and used for random priming during cDNA synthesis. Obtained cDNA was analysed by quantitative RT-PCR.

Quantitative Reverse Transcription PCR (qRT-PCR)

For quantitative analysis of *Drosophila* antimicrobial peptide gene expression, quantitative reverse transcription fluorescence PCR (qRT-PCR) was done using the double-stranded DNA dye SYBR Green (Bioline) in accordance with manufacturer's instructions. The following primer pairs were used: ***diptericin*** (*Dpt*, *CG12763*) sense, 5′-ACCGCAGTACCCACTCAATC-3′, antisense, 5′-CCCAAGTGCTGTCCATATCC-3′ ***attacin*** (*AttA*, *CG10146*) sense, 5′-CACAATGTGGTGGGTCAGG-3′, antisense, 5′-GGCACCATGACCAGCATT-3′ ***defensin*** (*Def*, *CG1385*) sense, 5′-TTCTCGTGGCTATCGCTTTT-3′, antisense, 5′-GGAGAGTAGGTCGCATGTGG-3′ ***metchnikowin*** (*Mtk*, *CG8175*) sense, 5′-TCTTGGAGCGATTTTTCTGG-3′; antisense, 5′-TCTGCCAGCACTGATGTAGC-3′ ***drosocin*** (*Dro*, *CG10816*) sense, 5′-CCATCGAGGATCACCTGACT-3′; antisense, 5′-CTTTAGGCGGGCAGAATG-3′ ***drosomycin*** (*Drs*, *CG10810*) sense, 5′-GTACTTGTTCGCCCTCTTCG-3′; antisense, 5′-CTTGCACACACGACGACAG-3′ ***ribosomal protein L4*** (*RpL1*, *CG5502*) sense, 5′-TCCACCTTGAAGAAGGGCTA-3′; antisense 5′-TTGCGGATCTCCTCAGACTT-3′.

The primer pairs were designed using Universal ProbeLibrary (Roche, https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp) to detect the desired gene transcripts, and supplied by Sigma. As a normalising gene, we used the ubiquitous *ribosomal protein L4* (*RpL1*) [31,32]. qRT-PCR analysis was done using the

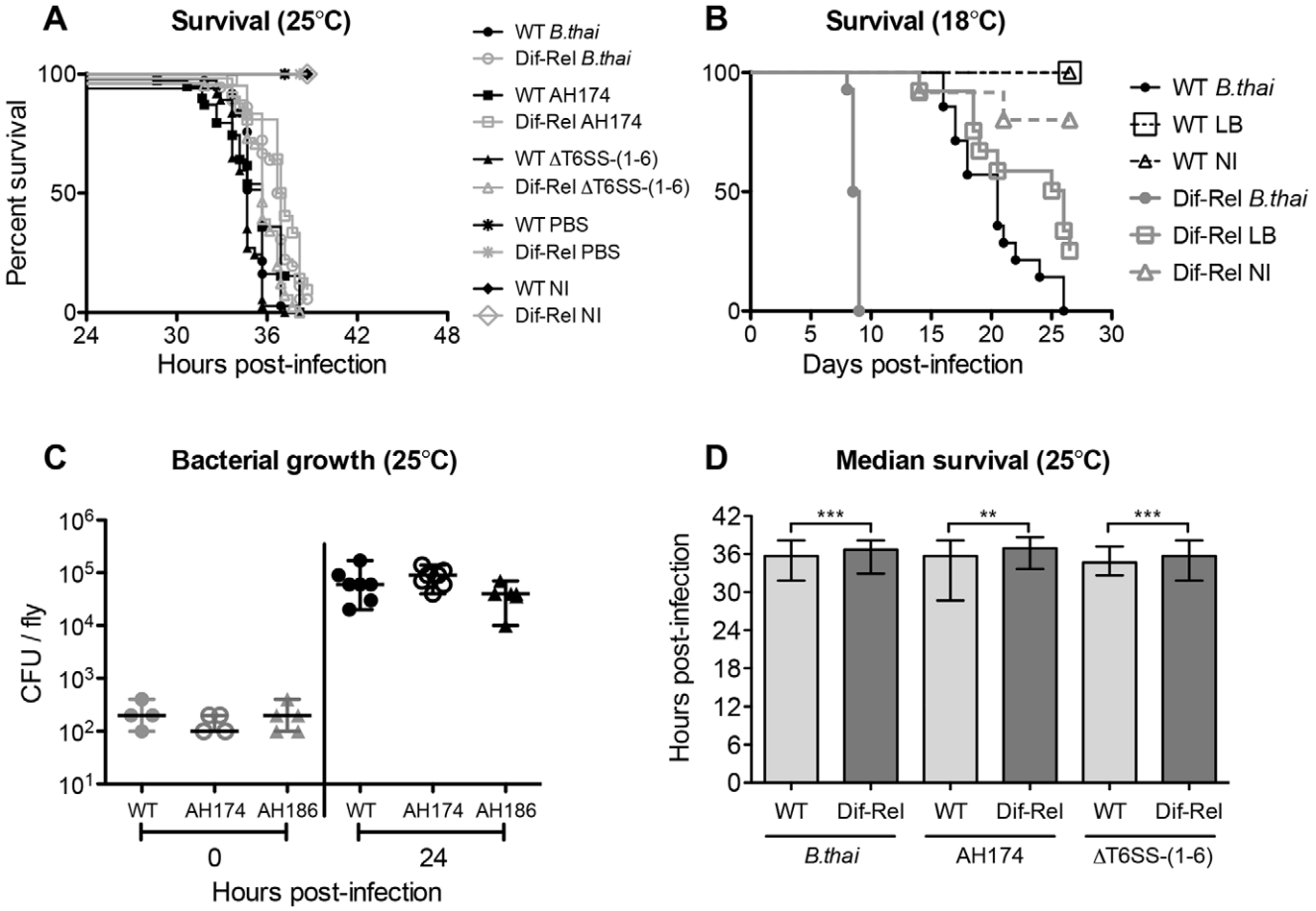

Figure 4. Type III (T3SS) and VI (T6SS) secretion systems are not required for virulence in *Drosophila*. (A) Survival of wild-type or *Dif; Rel* mutant flies infected with exponential-phase wild-type, T3SS-mutant (AH174) or T6SS-mutant *B. thailandensis*, maintained at 25°C, and counted at least every hour. (B) Median survival times from [A]. Statistical significance was determined using Mann-Whitney test; ** $p<0.002$ and *** $p<0.0005$. (C) Survival of wild-type and *Dif; Rel* mutant flies after infection with wild-type *B. thailandensis* E264 at 18°C. Under these conditions, *Dif; Rel* mutants were significantly shorter-lived. Statistical significance between the survival curves of infected WT and mutant flies was determined using Log-rank analysis (Mantel-Cox); $p<0.0001$. The data showing the WT *Drosophila* subset is the same as in [Fig. 2A]; all results shown here were obtained at the same time. (D) Proliferation of the T3SS mutant (AH174), and complemented AH186 mutant in WT *Drosophila*. Statistical significance of bacterial growth between time points was determined using Mann-Whitney test.
doi:10.1371/journal.pone.0049745.g004

Rotor-Gene 6000 (Corbett Life Science) and Rotor-Gene 6000 Series Software (Corbett Life Science).

B. thailandensis load in infected D. melanogaster

Infected *D. melanogaster* (1 male per sample) and controls were collected and homogenised in 100 µl of PBS at required time points. One tenth of each sample was processed into a series of dilutions of 1 in 10 in PBS; 4 dilutions were made in total. 10 µl of each incremental dilution was plated on a standard LB agar plate and kept at 37°C for 24 hours. Bacterial colonies were counted on a light microscope (Nikon). Finally, to obtain the approximate numbers of viable bacteria (CFU) per fly at a given time point of infection, individual bacterial counts were multiplied appropriately, e.g. the number of colonies obtained from the first dilution (1 in 10) was multiplied by 100. Obtained results were analysed using Prism (GraphPad Software).

Imaging

For imaging experiments, adult *Drosophila* males were treated in the same way as for survival assays, but were injected with GFP-labelled *B. thailandensis* E264 (medium dose OD_{600} of 0.1) or with dead pHrodo-conjugated *E. coli*, a rhodamine sensor of pH (pHrodo *E. coli* BioParticles®, Invitrogen). Controls were injected with PBS or uninjected (NI). Infected, injected or control flies were immobilised with the help of cyanoacrylate-based glue (Loctite), and imaged 6 or 24 h p.i. using a fluorescent (Leica) or confocal microscope (Leica TCS SP5) and capturing software (Leica Application Suite Advanced Fluorescence software). All images were processed using Adobe Photoshop CS5, and precisely the same adjustments were made to all images within an experiment.

Results

B. thailandensis E264 is pathogenic in *Drosophila melanogaster* and induces antimicrobial peptides

Burkholderia thailandensis E264 (*B. thailandensis*) is avirulent in people under normal conditions; however, it is highly pathogenic in wild-type (*Oregon-R*) *D. melanogaster* (5–10 days old). 100% of flies injected with *B. thailandensis* died reliably within 3.5 days of infection [Fig. 1A] and increasing bacterial dose resulted in more rapid mortality [Fig. S1A]. The survival assays were repeated several times using only the lowest bacterial dose (OD_{600} of 0.01).

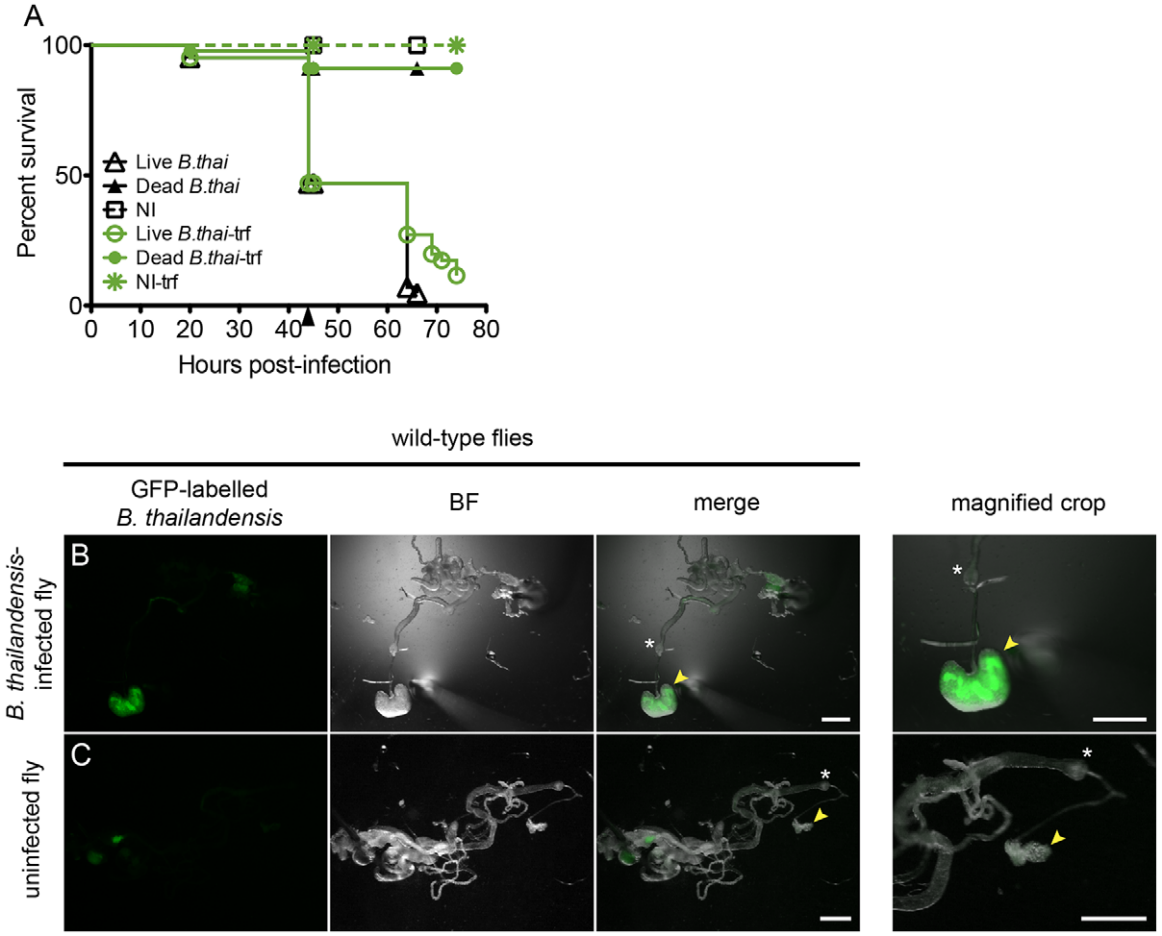

Figure 5. WT flies fed *B. thailandensis*-infected food are killed and have enlarged crop. (A) Survival of WT flies on infected food at 25°C. Flies kept on infected food died within 3.5 days after they were placed on this food. Second set of flies (trf) was kept on infected food and transferred to normal food, free of bacteria, at 44 hr (black arrowhead). The survival of the tranferred flies was slightly increased in comparison to the non-transferred group, but this difference was not significant. Controls were fed either food containing heat-killed *B. thailandensis* or no bacterium. The survival of the control groups was not affected. Sample size was at least 40 flies per condition. (B) Dissected gut of WT male *D. melanogaster* fed food infected with GFP-labelled *B. thailandensis*. The presence of the bacteria in the crop is confirmed by green fluorescence, which is visible only in the infected flies. (C) An uninfected control had a smaller crop. The crops of the infected and uninfected flies are shown at a higher magnification [magnified crop]. At least 3 flies were imaged per condition. Yellow arrowheads point to crop; white asterisks mark the proventriculus. Scale bars represent 500 μm.
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We also tested w^{1118} males (DrosDel isogenic background) to see if the effect of *B. thailandensis* infection was the same as it had been in *Oregon-R* flies. The survival data is consistent in both genotypes [data not shown]. Finally, this lethality required live bacteria: heat-killed *B. thailandensis* did not cause lethality [Fig. S1B].

We next wanted to test whether the observed lethality was accompanied by bacterial proliferation. We analysed *B. thailandensis* growth in infected flies by homogenising them in PBS at 0, 6 and 24 hours p.i. and counting viable bacterial colonies. *B. thailandensis* survived in the fly; an initial phase of low growth between 0 and 6 hours after infection was followed by rapid bacterial proliferation [Fig. 1B].

As an initial test of the immune response to *B. thailandensis*, we examined induction of antimicrobial peptides (AMPs) by this infection. *B. thailandensis* strongly induced all tested AMPs, including *Diptericin*, *Attacin*, *Drosocin*, *Drosomycin*, *Metchinkowin*, and *Defensin* [Fig. 1C]. Despite the strong induction, bacteria proliferated and infected flies died rapidly.

B. thailandensis E264 is thus a highly virulent pathogen in *Drosophila*, with a low dose (~250 CFU per fly) leading to rapid death of the host. For subsequent experiments, we have focused on the effects of the lowest verified infectious dose (OD_{600} of 0.01).

Temperature effect on survival of infected flies, and bacterial growth

We next investigated the role of temperature in this infection. Previous experiments had shown that the distantly-related *Burkholderia cepacia* was capable of killing flies at 18°C [28]. We observed that the *B. thailandensis* infection was dramatically slowed at 18°C: median survival time increases from 2 days at 25°C [Fig. 1A] to 20 days when infected files were kept at 18°C [Fig. 2A]. This effect was qualitatively similar to, but quantitatively larger than, the temperature effect seen in *Pseudomonas aeruginosa* infection [33]. This was accompanied by a dramatic increase in bacterial doubling time. Intriguingly, flies could be infected and maintained at 18°C, with bacterial numbers stable or only very slowly increasing; when these animals were shifted to 25°C, the

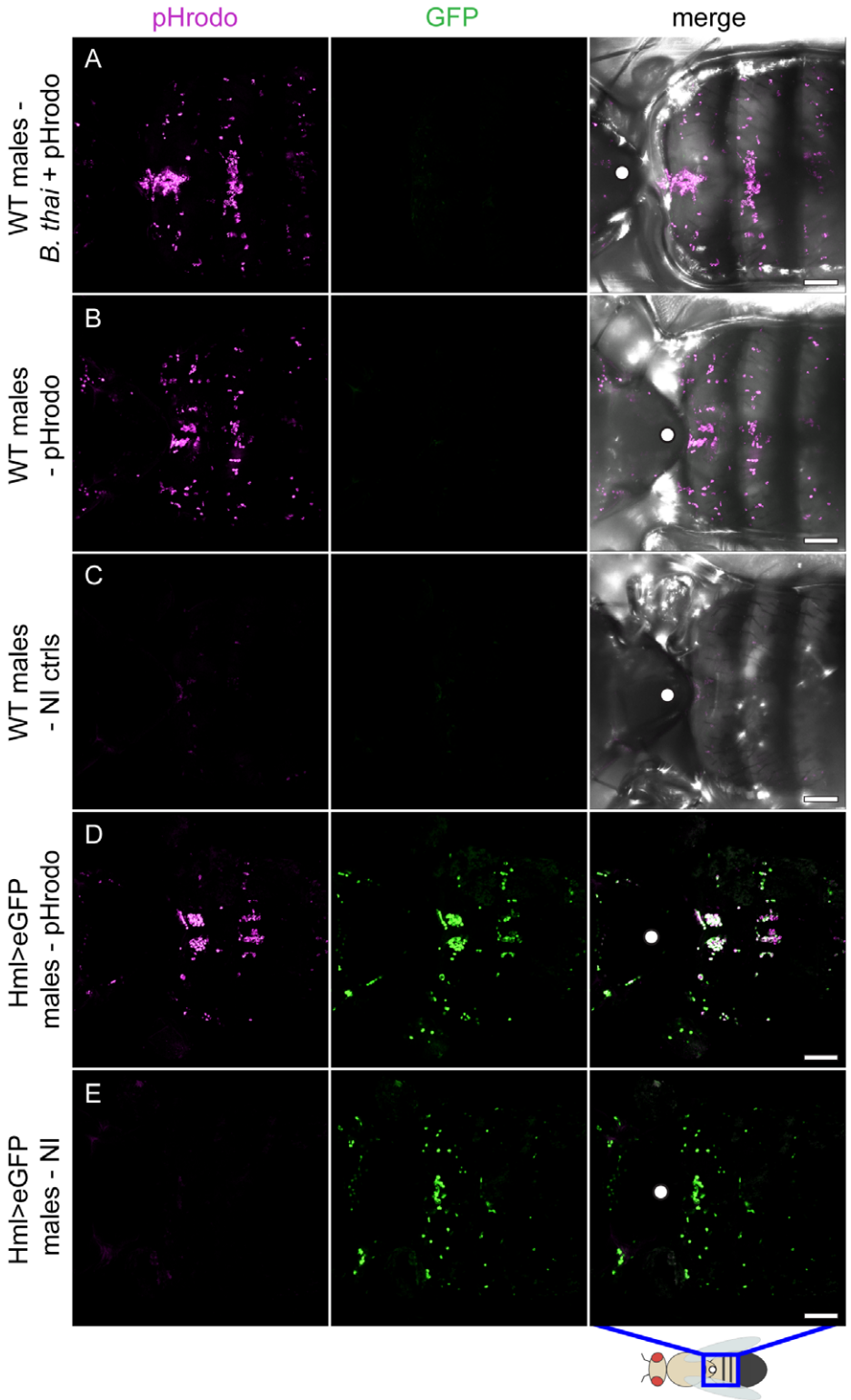

Figure 6. ***B. thailandensis*** **infection does not appear to affect phagocytic function of adult plasmatocytes.** (A) To examine the fate of haemocytes in this infection, WT males were infected with WT *B. thailandensis*, and 24 h later injected with pHrodo. Flies were imaged 4–5 h after pHrodo injection. The pHrodo beads were localised to haemocytes (magenta). (B) WT controls injected with pHrodo only. (C) Uninjected controls (NI). (D) pHrodo-injected flies expressing eGFP in a haemocyte-specific pattern (HmlΔGAL4, UAS-2xeGFP); at least 3 flies were imaged per condition. pHrodo is visible in magenta; co-localisation in white. (E) Untreated Hml>eGFP controls. The pattern of phagocytosed pHrodo was consistent with the pattern of haemocytes of NI flies that expressed eGFP in haemocytes. Since the pHrodo dye is bright fluorescent red only in an acidic environment, this result suggests that ~24 h before death, haemocytes of infected flies are functioning and visible (magenta). Scale bars represent 100 μm. The cartoon shows the dorsal side of *D. melanogaster*; the blue rectangle marks the area that was imaged; the white dot marks the notum.
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infection switched from chronic to acute [Fig. 2B], with bacterial numbers rapidly increasing [Fig. 2C] and causing the death of the host within one or two days of shifting to 25°C.

Sterile *B. thailandensis*-conditioned medium is lethal to the fly

B. pseudomallei causes pathology in part by the production of exotoxins [34,35]. In order to see whether some exotoxin might account for some or all of the lethality observed in this infection, we injected flies with sterile spent medium in which *B. thailandensis* had previously grown. *B. thailandensis* was grown overnight in LB at 37°C. The culture was spun at 2400×g for 4 minutes; supernatant was removed into a new tube and sterile-filtered using a 0.2 µm filter. To ensure that the sterile conditioned medium (CM) contained no live bacteria, a portion of the same CM that was injected into flies was plated on LB agar and kept at 37°C for 48 hours; no colonies grew (data not shown). As a control for this set of experiments, LB was kept overnight at 37°C alongside the incubating *B. thailandensis* culture, processed precisely the same way as the bacterial culture, and used for mock-infections. A portion of the sterile-filtered LB was also plated to prove that it had not been contaminated; no colonies grew at 37°C in 48 hours. When the sterile-filtered *B. thailandensis*-conditioned medium was injected into WT flies, it killed them as efficiently as live *B. thailandensis* although with a delayed kinetic [Fig. 3A]. The median time to death of flies infected with an overnight culture of live *B. thailandensis* was 46 h post-infection [Fig. 1A], while with *B. thailandensis*-CM it was approximately 69 h [Fig. 3A]. In contrast, neither *E. coli*-conditioned medium nor the overnight-treated LB was able to kill flies [Fig. 3A, S1C]. Heat treatment of *B. thailandensis*-conditioned medium was sufficient to eliminate its toxicity, and heat-killed whole *B. thailandensis* had no toxic effect [Fig. 3B, S1B]. *Drosophila* injected with either *B. thailandensis* culture grown overnight in LB or with one washed and resuspended in PBS died at an approximately the same rate; survival curves were not significantly different from each other (data not shown).

Despite being lethal to WT flies, *B. thailandensis*-conditioned medium did not induce a systemic immune response: *D. melanogaster* AMPs Drosocin and Attacin, which were strongly induced by infection with live bacteria, were not induced [Fig. 3C].

The inducible humoral immune response has differential effect on *B. thailandensis* infection depending on temperature

We had seen that infection with live *B. thailandensis* strongly stimulated antimicrobial peptide expression in the fly. As the AMP response is the most important determinant of survival in most bacterial infections in the fly, we tested the ability of flies lacking *Dif* and *Rel*, the two most prominent transcriptional effectors of this response, to survive infection with *B. thailandensis*. These animals are incapable of producing antimicrobial peptides in response to immune challenge [36,37]. *Dif; Rel* double mutants exhibited no increase in susceptibility when infected with exponential-phase *B. thailandensis* at 25°C [Fig. 4A, 4B, Fig. S2A]. In fact, *Dif; Rel* mutants were consistently very slightly longer-lived than wild-type animals (an effect only detectable by counting dead flies at extremely frequent intervals); though this effect was consistently seen, and cannot be explained by different times of infection, its origin and importance is unclear.

Finally, we examined the interaction between environmental (temperature) effects and host genotype by infecting *Dif; Rel* mutants at 18°C. In contrast to the effect seen at 25°C, *Dif; Rel* mutants infected at 18°C died much faster than wild-type flies (median survival time = 8 days) [Fig. 4C].

B. thailandensis E264 Type III and Type VI secretion systems do not play a role in virulence to *D. melanogaster*

Having established that the humoral immune response is not critical in this infection at 25°C, we examined bacterial virulence mutants in the hope of finding some key effector of pathogenesis.

We tested the virulence of Bsa Type III secretion system mutant (AH174) and the complemented mutant (AH186); the AH174 mutant has a strong virulence defect in mice [18]. The mutation had no effect on the survival of *B. thailandensis*-infected flies at 25°C [Fig. 4A, 4B, Fig. S2B] (survival data for AH186 are not shown but were identical to both AH174 and wild-type E264). The same was true for *Dif; Rel* mutant flies. In wild-type flies, the growth of the $T3SS_{Bsa}$ mutant was not significantly different from WT *B. thailandensis* [Fig. 4D].

We next tested the role of the Type VI secretion systems in virulence in *Drosophila*. Schwarz and colleagues observed that *B. thailandensis* lacking Type VI secretion system number 5 (ΔT6SS-5) had reduced virulence in mice, while T6SS-1 was important in *B. thailandensis* survival in competition with other Gram-negative bacteria, such as *Pseudomonas putida* and *Serratia proteamaculans* [29]. In flies, we found that, as with the Type III mutant above, a *B. thailandensis* mutant lacking all five Type VI secretion systems, ΔT6SS-(1–6), exhibited wild-type virulence at 25°C in wild-type animals and *Dif; Rel* mutants [Fig. 4A, 4B, Fig. S2C].

Food infected with *B. thailandensis* E264 kills wild-type flies

In order to examine the effects of oral infection with *B. thailandensis*, we inoculated a potato-milk-fructose *Drosophila* food mix with the WT GFP-expressing strain, AH183 [18]. AH183 was tested in a survival assay to ensure that its virulence was similar to that of wild-type E264 [Fig. S3]. Flies transferred onto this food apparently remained healthy for at least 24 hours, but by 48 hours, 50% of the flies had died [Fig. 5A]. Flies that were surviving at this time were transferred to fresh uninfected food; these animals nonetheless succumbed to the infection. Oral infection killed flies with similar kinetics to infection by direct introduction of bacteria into the haemolymph. On dissection, GFP-expressing bacteria were clearly present in the gut [Fig. 5B, 5C]; in particular, the crop of these animals tended to be dramatically distended and often contained large amounts of GFP-positive material. No GFP-positive bacteria could be detected outside the gut in any animal at any stage of oral infection, and upon dissection the gut itself was not visibly breached by the infection.

D. melanogaster haemocytes function is not impaired by *B. thailandensis*

Some infections in *Drosophila* inhibit the bactericidal phagocyte system [24,38–41]. To understand what effect *B. thailandensis* has on haemocytes we used pHrodo-labelled *E. coli* BioParticles® (pHrodo). pHrodo is rhodamine-based dye that is conjugated to dead bacteria as a probe for phagocytosis; it is red fluorescent only at a low pH, such as that found in phagocytic vesicles. This fluorogenic feature allows specific imaging of phagocytosis and also, in this case, confirmed that injected pHrodo-labelled bacteria were internalised by haemocytes of *B. thailandensis*-infected *D. melanogaster* approximately 24 h before the host was killed by this infection [Fig. 6A]. The obtained data shows that the distribution of pHrodo-containing haemocytes in infected flies is comparable

to that of flies injected only with pHrodo, but with no bacteria [Fig. 6B]. Untreated controls were imaged at the same time as infected flies; no fluorescence was visible, only slight auto-fluorescence was noted [Fig. 6C]. All infected and control flies were imaged in a GFP channel. In addition, we used *D. melanogaster* expressing eGFP in a haemocyte-specific manner, *HmlΔGAL4, UAS-2xeGFP*, as a control to show the colocalisation of pHrodo and haemocytes [Fig. 6D]; untreated controls were also imaged [Fig. 6E]. Attempts to localize injected *B. thailandensis* using the GFP-expressing strain were stymied by inconsistent localization (data not shown).

Based on our results, *B. thailandensis* infection in *D. melanogaster* had not destroyed the phagocytic capabilities of fly haemocytes approximately 24 h before death.

Discussion

In this study, we tested *B. thailandensis* as a potential *D. melanogaster* pathogen and found that the bacterium was highly virulent in the fly. This bacterium is mostly avirulent in humans, but exceptions have been recorded where *B. thailandensis* infection resulted in melioidosis-like symptoms [22,23]. *Drosophila* has been shown to be a genetically tractable model in other infections [24–26,42].

B. thailandensis survives and multiplies in infected flies. The bacterium grows well at 25–37°C [9] and when injected into *Drosophila*, it multiplies until the time of the host's death. The lethal dose of *B. thailandensis* is approximately 250 CFU per fly. Growth between 0 h and 6 h post-infection is slow and statistically insignificant; however, the bacterial burden at 24 h post-infection was significantly higher in comparison to that obtained at 6 h p.i.

Although *B. thailandensis* infection induces expression of *Drosophila* AMPs, the bacterium kills its host within 48 hours, and *Drosophila* mutants that cannot produce AMPs exhibit no increase in susceptibility to the infection at 25°C. This result suggests that *B. thailandensis* may be resistant to AMPs, much as *B. pseudomallei* is resistant to human defensin HNP-1 *in vitro* [43]. In this study *B. pseudomallei*, but not *S. typhimurium* or *E. coli*, was resistant to HNP-1 [43]. Other possible explanations for this observation include bacterial disruption of AMP production at a post-transcriptional level, or the persistence of bacteria in some sheltered compartment (for example, the phagocyte). The fact that injected *B. thailandensis* exhibited no consistent anatomical localization and did not disrupt the activity of the bactericidal phagocyte system against *E. coli* reduce the likelihood of this last possibility but do not completely preclude it.

Sterile *B. thailandensis*-conditioned medium, completely free of live bacteria, proved to be as pathogenic in the fly as live bacteria. This result suggests that *B. thailandensis* secretes an exotoxin. The exotoxin might share similarity to toxins secreted by *B. pseudomallei* [34,35]. Although the *B. thailandensis* 'toxin' alone kills, the bacterial culture washed and resuspended in PBS, and thus free of the 'toxin', kills faster in comparison with sterile bacteria-conditioned medium, implying that the exotoxin present in spent medium cannot be the sole effector of bacterial pathogenicity. Heat-treatment eliminated the activity of this toxin, suggesting that it may be proteinaceous (and is in any case unlikely to be a stable small molecule). The identity of this toxin is of clear interest.

The mechanism of reduced virulence of the *B. thailandensis* at low temperature (18°C) is not yet clear. One possibility is that the activity of the implied exotoxin may be reduced at low temperatures; this effect has been observed previously with ricin and shiga toxin [44,45]. In this regard, it may be relevant that, in addition to the *B. thailandensis* exotoxin for which we provide evidence here, *B. pseudomallei* produces exotoxin and proteases [34,35].

Conversely, the observation that *Dif; Rel* mutants do exhibit significant immune compromise relative to wild-type animals at 18°C suggests that either antimicrobial peptides might be more efficient at cooler temperatures or the bacterial surface might be changed in some way at lower temperature, rendering it more sensitive to the effects of antimicrobial peptides. Speculating further, it might be possible that at 18°C *B. thailandensis*' reproduction and dynamics are slower, and any potential cellular invasion might occur at a reduced pace, thus giving the AMPs more time to be efficient. Whereas in the *Dif; Rel* mutants, the bacterial replication and dynamics might be the same as in wild-type *Drosophila*, but the absence of AMPs in the immunocompromised mutants might result in increased virulence. Finally, this effect and the temperature effect on bacterial virulence might be two sides of the same coin, with a complex interaction between specific bacterial virulence factors and relative activity levels of different immune effectors giving rise to the observed dramatic changes in infection dynamics at different temperatures.

Although neither the T3SS nor T6SS appear not to affect the function of *Drosophila* haemocytes, our observation was made only qualitatively: phagocytic index was not quantified as had been done in a previous study, in which *P. aeruginosa* T3SS was shown to interfere with haemocyte phagocytic function [38]. It remains possible that the T3SS or the T6SS mutants might exhibit a detectable change in virulence if assayed in a more sensitive fashion, such as competitive index as previously shown for closely related Bcc species [27,29].

The fact that *B. thailandensis* persisted in the gut and ultimately killed the fly after oral infection is particularly intriguing given the recent observation that pesticide-degrading *Burkholderia* strains are specific beneficial endosymbionts of several important phytophagous insects [46]. We were unable to detect *B. thailandensis* crossing the gut barrier; that said, it is not clear whether its deleterious effects in the gut are due to toxin secretion acting on the host, nutrient effects, or undetected systemic infection.

One aim of this study was to establish whether infection of *D. melanogaster* with *Burkholderia thailandensis* could be a useful model for mammalian melioidosis. Though flies are rapidly killed by *B. thailandensis*, the fact that neither Type III nor Type VI secretion systems appear to be required for virulence in the fly suggests that many virulence factors will not be conserved in this host, potentially limiting its general utility. Nonetheless, several aspects of this infection, including the presence of an apparent heat-labile exotoxin and the ability to kill flies by feeding, and the previously-observed association of other *Burkholderaciae* with insects, represent intriguing avenues for further study.

Supporting Information

Figure S1 Control infections with WT or heat-killed *B. thailandensis*, or with conditioned medium. (A) Infected *D. melanogaster* was killed in a dose-dependent manner. The result is based on a single experiment; n = 19 flies per genotype per condition. Three infectious doses were tested: OD600 = 0.01 (low), 0.1 (medium), and 1 (high). Mock-infected (PBS) and untreated (NI) controls were alive for the whole duration of this experiment. (B) Heat-killed (H/K) *B. thailandensis* was avirulent in WT males at 25°C. High dose of *B. thailandensis* was OD600 of 1; low OD600 of 0.01; n = min. 16 flies per condition. (C) *E. coli*-conditioned medium (*E.coli*-CM) was not infectious at 25°C in comparison to that of *B. thailandensis* (*B.thai*-GFP-CM); n = min. 14 flies per condition.
(TIF)

Figure S2 Infections of WT and mutant *Drosophila* with wild-type, T3SS or T6SS *B. thailandensis*. Survival curves isolated from [Fig. 4A] showing data of WT and *Dif; Rel* mutant *D. melanogaster* infected with (A) WT *B. thailandensis*, (B) T3SS mutant, or (C) T6SS mutant, at 25°C.
(TIF)

Figure S3 Control infections with GFP-labelled *B. thailandensis*. Flies infected with GFP-labelled *B. thailandensis* died within 2 days p.i., which is comparable to infections with non-GFP-labelled *B. thailandensis*; n = 20 flies per condition.
(TIF)

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Author Contributions

Conceived and designed the experiments: MP MSD. Performed the experiments: MP MSD. Analyzed the data: MP MSD. Wrote the paper: MP MSD.

References

1. Cheng AC, Currie BJ (2005) Melioidosis: epidemiology, pathophysiology, and management. Clinical Microbiology Reviews 18: 383–416.
2. Smith MD, Wuthiekanun V, Walsh AL, White NJ (1995) Quantitative recovery of Burkholderia pseudomallei from soil in Thailand. Transactions of the Royal Society of Tropical Medicine and Hygiene 89: 488–490.
3. Chaowagul W, White NJ, Dance DA, Wattanagoon Y, Naigowit P, et al. (1989) Melioidosis: a major cause of community-acquired septicemia in northeastern Thailand. Journal of Infectious Diseases 159: 890–899.
4. White NJ (2003) Melioidosis. Lancet 361: 1715–1722.
5. Currie BJ, Fisher DA, Howard DM, Burrow JN, Selvanayagam S, et al. (2000) The epidemiology of melioidosis in Australia and Papua New Guinea. Acta Tropica 74: 121–127.
6. Chaowagul W, Suputtamongkol Y, Dance DA, Rajchanuvong A, Pattara-arechachai J, et al. (1993) Relapse in melioidosis: incidence and risk factors. Journal of Infectious Diseases 168: 1181–1185.
7. Currie BJ, Fisher DA, Howard DM, Burrow JN, Lo D, et al. (2000) Endemic melioidosis in tropical northern Australia: a 10-year prospective study and review of the literature. Clinical Infectious Diseases 31: 981–986.
8. Suputtamongkol Y, Chaowagul W, Chetchotisakd P, Lertpatanasuwun N, Intaranongpai S, et al. (1999) Risk factors for melioidosis and bacteremic melioidosis. Clinical Infectious Diseases 29: 408–413.
9. Brett PJ, DeShazer D, Woods DE (1997) Characterization of Burkholderia pseudomallei and Burkholderia pseudomallei-like strains. Epidemiology and Infection 118: 137–148.
10. Pilatz S, Breitbach K, Hein N, Fehlhaber B, Schulze J, et al. (2006) Identification of Burkholderia pseudomallei genes required for the intracellular life cycle and in vivo virulence. Infection and Immunity 74: 3576–3586.
11. Stevens JM, Ulrich RL, Taylor LA, Wood MW, DeShazer D, et al. (2005) Actin-binding proteins from Burkholderia mallei and Burkholderia thailandensis can functionally compensate for the actin-based motility defect of a Burkholderia pseudomallei bimA mutant. Journal of Bacteriology 187: 7857–7862.
12. Rotz LD, Khan AS, Lillibridge SR, Ostroff SM, Hughes JM (2002) Public health assessment of potential biological terrorism agents. Emerging Infectious Diseases 8: 225–230.
13. Schweizer HP, Peacock SJ (2008) Antimicrobial Drug–Selection Markers for Burkholderia pseudomallei and B. mallei. Emerging Infectious Diseases 14: 1689–1692.
14. Vorachit M, Lam K, Jayanetra P, Costerton JW (1993) Resistance of Pseudomonas pseudomallei growing as a biofilm on silastic discs to ceftazidime and co-trimoxazole. Antimicrobial Agents and Chemotherapy 37: 2000–2002.
15. Brett PJ, DeShazer D, Woods DE (1998) Burkholderia thailandensis sp. nov., a Burkholderia pseudomallei-like species. International Journal of Systematic Bacteriology 48: 317–320.
16. Kim HS, Schell MA, Yu Y, Ulrich RL, Sarria SH, et al. (2005) Bacterial genome adaptation to niches: divergence of the potential virulence genes in three Burkholderia species of different survival strategies. BMC Genomics 6: 174.
17. Yu Y, Kim HS, Chua HH, Lin CH, Sim SH, et al. (2006) Genomic patterns of pathogen evolution revealed by comparison of Burkholderia pseudomallei, the causative agent of melioidosis, to avirulent Burkholderia thailandensis. BMC Microbiology 6: 46.
18. Haraga A, West TE, Brittnacher MJ, Skerrett SJ, Miller SI (2008) Burkholderia thailandensis as a model system for the study of the virulence-associated type III secretion system of Burkholderia pseudomallei. Infection and Immunity 76: 5402–5411.
19. Wiersinga WJ, de Vos AF, de Beer R, Wieland CW, Roelofs JJTH, et al. (2008) Inflammation patterns induced by different Burkholderia species in mice. Cellular Microbiology 10: 81–87.
20. Kespichayawattana W, Rattanachetkul S, Wanun T, Utaisincharoen P, Sirisinha S (2000) Burkholderia pseudomallei Induces Cell Fusion and Actin-Associated Membrane Protrusion: a Possible Mechanism for Cell-to-Cell Spreading. Infection and Immunity 68: 5377–5384.
21. Stevens MP, Stevens JM, Jeng RL, Taylor LA, Wood MW, et al. (2005) Identification of a bacterial factor required for actin-based motility of Burkholderia pseudomallei. Molecular Microbiology 56: 40–53.
22. Lertpatanasuwan N, Sermsri K, Petkaseam A, Trakulsomboon S, Thamlikitkul V, et al. (1999) Arabinose-positive Burkholderia pseudomallei infection in humans: case report. Clinical Infectious Diseases 28: 927–928.
23. Glass MB, Gee JE, Steigerwalt AG, Cavuoti D, Barton T, et al. (2006) Pneumonia and septicemia caused by Burkholderia thailandensis in the United States. Journal of Clinical Microbiology 44: 4601–4604.
24. Dionne MS, Ghori N, Schneider DS (2003) Drosophila melanogaster is a genetically tractable model host for Mycobacterium marinum. Infection and Immunity 71: 3540–3550.
25. Brandt SM, Dionne MS, Khush RS, Pham LN, Vigdal TJ, et al. (2004) Secreted Bacterial Effectors and Host-Produced Eiger/TNF Drive Death in a Salmonella-Infected Fruit Fly. PLoS Biology 2: e418.
26. Needham AJ, Kibart M, Crossley H, Ingham PW, Foster SJ (2004) Drosophila melanogaster as a model host for Staphylococcus aureus infection. Microbiology 150: 2347–2355.
27. Castonguay-Vanier J, Vial L, Tremblay J, Déziel E (2010) Drosophila melanogaster as a model host for the Burkholderia cepacia complex. PLoS ONE 5: e11467.
28. Schneider DS, Ayres JS, Brandt SM, Costa A, Dionne MS, et al. (2007) Drosophila eiger mutants are sensitive to extracellular pathogens. PLoS Pathogens 3: e41.
29. Schwarz S, West TE, Boyer F, Chiang WC, Carl MA, et al. (2010) Burkholderia type VI secretion systems have distinct roles in eukaryotic and bacterial cell interactions. PLoS Pathogens 6: e1001068.
30. Sarkar-Tyson M, Smither SJ, Harding SV, Atkins TP, Titball RW (2009) Protective efficacy of heat-inactivated B. thailandensis, B. mallei or B. pseudomallei against experimental melioidosis and glanders. Vaccine 27: 4447–4451.
31. Cho Y, Griswold A, Campbell C, Min K-T (2005) Individual histone deacetylases in Drosophila modulate transcription of distinct genes. Genomics 86: 606–617.
32. Ranz JM, Casals F, Ruiz A (2001) How malleable is the eukaryotic genome? Extreme rate of chromosomal rearrangement in the genus Drosophila. Genome Research 11: 230–239.
33. Apidianakis Y, Rahme LG (2009) Drosophila melanogaster as a model host for studying Pseudomonas aeruginosa infection. Nature protocols 4: 1285–1294.
34. Haase A, Janzen J, Barrett S, Currie B (1997) Toxin production by Burkholderia pseudomallei strains and correlation with severity of melioidosis. Journal of Medical Microbiology 46: 557–563.
35. Häussler S, Nimtz M, Domke T, Wray V, Steinmetz I (1998) Purification and characterization of a cytotoxic exolipid of Burkholderia pseudomallei. Infection and Immunity 66: 1588–1593.
36. Hedengren M, Åsling B, Dushay MS, Ando I, Ekengren S, et al. (1999) Relish, a central factor in the control of humoral but not cellular immunity in Drosophila. Molecular Cell 4: 827–837.
37. Rutschmann S, Jung AC, Hetru C, Reichhart JM, Hoffmann JA, et al. (2000) The Rel Protein DIF Mediates the Antifungal but Not the Antibacterial Host Defense in *Drosophila*. Immunity 12: 569–580.
38. Avet-Rochex A, Bergeret E, Attree I, Meister M, Fauvarque M-O (2005) Suppression of Drosophila cellular immunity by directed expression of the ExoS toxin GAP domain of Pseudomonas aeruginosa. Cellular Microbiology 7: 799–810.
39. Chamilos G, Lewis RE, Hu J, Xiao L, Zal T, et al. (2008) Drosophila melanogaster as a model host to dissect the immunopathogenesis of zygomycosis. PNAS 105: 9367–9372.
40. Limmer S, Haller S, Drenkard E, Lee J, Yu S, et al. (2011) Pseudomonas aeruginosa RhlR is required to neutralize the cellular immune response in a Drosophila melanogaster oral infection model. PNAS 108: 17378–17383.
41. Mansfield BE, Dionne MS, Schneider DS, Freitag NE (2003) Exploration of host-pathogen interactions using Listeria monocytogenes and Drosophila melanogaster. Cellular Microbiology 5: 901–911.
42. D'Argenio DA, Gallagher LA, Berg CA, Manoil C (2001) Drosophila as a model host for Pseudomonas aeruginosa infection. Journal of Bacteriology 183: 1466–1471.
43. Jones AL, Beveridge TJ, Woods DE (1996) Intracellular survival of Burkholderia pseudomallei. Infection and Immunity 64: 782–790.

44. Mallard F, Antony C, Tenza D, Salamero J, Goud B, et al. (1998) Direct pathway from early/recycling endosomes to the Golgi apparatus revealed through the study of shiga toxin B-fragment transport. The Journal of cell biology 143: 973–990.
45. van Deurs B, Petersen OW, Olsnes S, Sandvig K (1987) Delivery of internalized ricin from endosomes to cisternal Golgi elements is a discontinuous, temperature-sensitive process. Experimental Cell Research 171: 137–152.
46. Kikuchi Y, Hayatsu M, Hosokawa T, Nagayama A, Tago K, et al. (2012) Symbiont-mediated insecticide resistance. PNAS 109: 8618–8622.